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CONTENTS

R. G. HURD: The Effect of pH and Bicarbonate Ions on the Uptake of Salts by Disks of Red Beet 159

G. E. BLACKMAN, R. S. BRUCE, and K. HOLLY: Studies in the Principles of Phytotoxicity. V .Interrelationships between Specific Differences in Spray Retention and Selective Toxicity 175

S. H. CROWDY, D. RUDD JONES, and A. V. WITT: The Translocation of Sulphonamides in Higher Plants 206

F. A. L. CLOWES: Protein Synthesis in Root Meristems 229

ELIZABETH E. HARDMAN and W. MARY CROMBIE: Fat Metabolism in Germinating *Citrullus vulgaris*. Part II . . . . . [illegible]

W. MARY CROMBIE and ELIZABETH E. HARDMAN: Fat Metabolism in the West African Oil Palm (*Elaeis guineensis*). Part III. Fatty Acid Formation in the Maturing Exocarp 247

W. MARY CROMBIE: Fatty Acids in Chloroplasts and Leaves . . . 254

R. SCOTT RUSSELL and HELEN M. SQUIRE: The Absorption and Distribution of Strontium in Plants. I. Preliminary Studies in Water Culture . 262

J. V. POSSINGHAM and R. BROWN: The Nuclear Incorporation of Iron and its Significance in Growth 277

F. C. STEWARD and R. G. S. BIDWELL: Nitrogen Metabolism, Respiration, and Growth of Cultured Plant Tissue. Part IV 285

A. BOOTH: Non-hormonal Growth Promotion Shown by Aqueous Extracts 306

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The Effect of pH and Bicarbonate Ions on the Uptake of Salts by Disks of Red Beet

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SUMMARY

1. The uptake of potassium chloride by disks of beetroot in solutions at different pH values has been followed over periods of several days.

2. It was found that, as the pH was raised from approximately 6 to 9 the rate of uptake of potassium from 0·0024 M. potassium chloride increased, and there was a corresponding increase in the amount of potassium held by the disks at saturation. Both the rate of uptake and the final content of potassium in disks kept in solutions at pH 8·5 were frequently double those at pH 6.

3. The rate of uptake of chloride ions was only slightly affected by the pH value of the medium. The final chloride content was approximately the same in disks kept at different pH values.

4. The above 'pH effect' on potassium uptake has been shown to be related to the uptake of bicarbonate ions present in the alkaline solution; the additional potassium ions accumulated accompanying the bicarbonate anions taken up.

5. The bicarbonate ions accumulated are converted primarily to malic acid.

INTRODUCTION

JACOBSON *et al* (1950) have put forward a basic scheme for the mechanism of salt uptake represented by the following equations:

$$Z^+ + RH \rightleftharpoons RZ + H^+ \quad (1)$$

$$A^- + R'OH \rightleftharpoons R'A + OH^- \quad (2)$$

They showed that, in accordance with equation (1), from the law of mass action, as the H^+ ion concentration in the medium surrounding their excised barley roots was increased so also was the loss of cations from the roots. They did not investigate the validity of equation (2), and this was the original purpose of the work described here. It was postulated that as the pH value was raised, increasing concentrations of hydroxyl ions would progressively diminish the uptake of the other anions in solution. This method of attempting to confirm equation (2) was preferred to a method analogous to the one used by Jacobson for cations because it seemed preferable to measure the uptake of ions rather than make an assessment of rates of leakage at relatively extreme pH values. There has been some criticism of this latter form of experiment since some of the pH values employed by Jacobson are known to be injurious to plant

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tissues. This may well result in an increased passive leakage of ions into solution which cannot be differentiated from those lost by exchange with H^+ ions (Nielsen and Overstreet, 1955).

Much work has been published on the effect of the pH of the medium on the uptake of salt by plant tissues (e.g. Olsen, 1953; Nielsen and Overstreet, 1955; Hoagland and Broyer, 1940). In general pH in itself does not appear to profoundly influence salt uptake; the indications are that the rate of uptake of both cations and anions is virtually independent of the pH value over the range pH 5 to 8, and gradually diminishes on either side of these limits. Frequently investigators have employed buffered solutions in an attempt to maintain constant pH values over the uptake period, but many of these 'buffer salts' are themselves accumulated by the plant tissues thus complicating the interpretation of the results. Steward and Preston (1941) investigated phosphate and bicarbonate buffer systems and showed that these may have a considerable influence on the pattern of uptake of salts by potato tissue. The primary amine tris-hydroxymethyl-aminomethane when combined with sulphuric acid acts as a buffer and it was anticipated from the work of Gomori (1946, 1948) and the inert structure of the molecule, that it would not interfere significantly with salt absorption. Preliminary experiments showed that insignificant amounts of the buffer were accumulated and that it had very little effect on the uptake of salt by beet; it does however, considerably, affect the uptake of salt by potato (Hurd, 1956).

METHODS AND MATERIALS

The root tissue of the red beetroot (*Beta vulgaris*) variety Crimson Globe was used. Cylinders of the tissue were cut with a 7·5 mm. diameter cork-borer and these were then sliced in a hand microtome to produce disks 0·8 mm. thick. The slices were washed in running tap water for 2 or 3 hours after cutting, and then placed in aerated glass distilled water which was changed daily for 3 to 6 days. This treatment caused the development of an absorptive capacity in the tissue prior to the experimental period (Stiles and Dent, 1946).

The uptake experiments were performed in boiling-tubes ($8'' \times 1\frac{1}{2}''$) containing 40 ml. of solution and 30 disks (approximately 1 g. fresh weight). A capillary tube reaching to the bottom of each boiling-tube provided approximately 6 l. of air per hour which was sufficient to keep the disks circulating. Rows of such tubes were fitted in a metal stand and were placed in a constant temperature bath at 25° C. The air was first humidified by passing through distilled water at 25° C. Condensation occurred in the air distributing manifold and this was allowed to run off into spare tubes. Over a period of 24 hours (the maximum period that any one solution was in a tube) the change in volume of the solution in the experimental tubes was less than 3 per cent. Even so controls were run using solutions without disks and these solutions were subsequently used as standards in the analyses. After each uptake period the entire solution was decanted from each tube for subsequent analysis and replaced by a fresh 40 ml. of medium.

In order to determine the uptake of salt accurately from this relatively large volume of solution the concentration was fixed at a low level (0·0024 M.) in the early experiments. Four replicates were employed in all the experiments. Potassium determinations were made with a flame photometer; chloride determinations by an electrometric method (Furman and Low, 1935). In the latter a comparison was made between the potential developed at the surface of two Ag/AgCl electrodes, one in a solution of known chloride concentration and the other in the unknown solution of potassium chloride.

pH measurement was made with a glass electrode in conjunction with a Cambridge pH meter.

The buffer 'tris' was used at a concentration of 0·01 M. and brought to the required pH with sulphuric acid. This combination is subsequently referred to as tris sulphate.

Bicarbonate estimation. The method used was either to measure manometrically the amount of carbon dioxide released from a known volume of medium containing bicarbonate on adding mineral acid, or to use ^{14}C-labelled bicarbonate and determine the change in radioactivity with time.

Methods employed using radioactive bicarbonate. Radioactive 0·0012 M. sodium bicarbonate solutions were prepared with an activity of about 0·2 mC/litre. The deposit from 0·1 ml. of this solution when spread on a planchet and placed 1 mm. from the end window counter had an approximate activity of 900 CPM. The amount of active bicarbonate accumulated in the tissue was estimated by counting an aliquot from an 80 per cent. alcoholic extract of the disks which had been evaporated to dryness on a planchet. The high rate of exchange over the uptake period between active carbon dioxide and inactive CO_2 respired from the disks and collected from the atmosphere made it impossible to calculate with sufficient accuracy the uptake of radioactive bicarbonate by the disks from the loss of activity of the solution. The activities of the initial solutions were obtained by converting the contained bicarbonate to the more stable carbonate using an equivalent amount of sodium hydroxide, before exposing the solutions to the air. In this form dried preparations retained their initial activity for at least 24 hours when left in a desiccator. (Experiments showed that, once dry, bicarbonate deposits could also be kept in this way.) The deposits were so small (0·001 g.) as to be well within the infinite thinness limit and, apart from an allowance for the background count, no corrections were made on the count obtained.

RESULTS

The effect of pH value of the solution on salt uptake. Tris has an effective buffer range of pH 6·0 to 9·0; experiments using this buffer were therefore confined within these limits. A typical experiment employing buffered solutions had the following treatments:

1. 0·0024 M. KCl and tris SO_4 —pH 8·5;
2. 0·0024 M. KCl and tris SO_4 —pH 6·1;
3. 0·0024 M. KCl unbuffered—approximately pH 6·0.

TABLE I

Uptake of 0·0024 M. KCl (μeq./g.f.w.) by beet disks over a 72-hour period at 25° C. Solutions buffered with tris SO_4 at pH 8·5 and pH 6·1

Uptake period in hrs.	pH 8·5			pH 6·1			pH 6·0, no buffer		
	K^+	Cl^-	Final pH	K^+	Cl^-	Final pH	K^+	Cl^-	Final pH
3	13·0	1·5	8·0	7·0	3·5	5·8	7·5	4·0	5·6
12	48·0	10·0	8·1	24·0	11·5	5·7	20·0	10·0	5·5
24	94·0	31·0	8·1	47·0	30·0	5·3	37·0	24·0	4·3
36	134·0	45·0	7·9	63·0	40·0	5·3	50·0	33·0	4·9
48	175·0	52·0	7·9	78·0	50·0	5·1	65·0	43·0	4·7
72	233·0	70·0	8·0	94·0	68·0	5·6	85·0	63·0	5·2

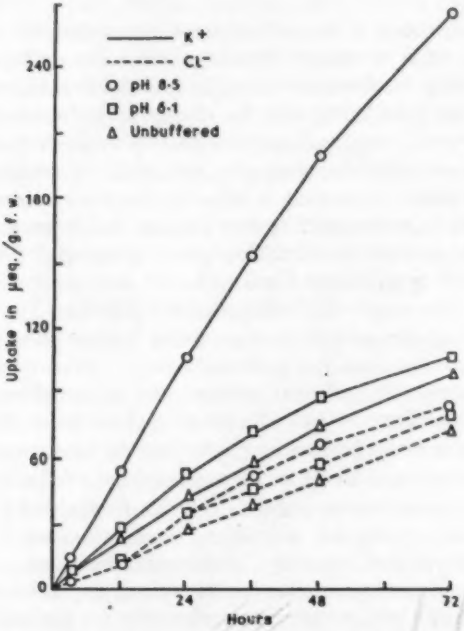

FIG. 1. Amounts of K^+ and Cl^- (μeq./g.f.w.) absorbed from 0·0024 M. KCl at different pH values at 25° C. by disks of red beetroot.

The solution containing buffer at pH 6·1 was poorly buffered, but it had approximately the same pH as the solution without the buffer. By comparing uptake from these two solutions, possible effects of the buffer itself on salt uptake could be detected. The observations from the results of two separate experiments given in Table I and Fig. 1 are (1) The uptake of potassium from the solution at pH 8·5 was more than double that from solutions at pH 6·0—6·1. (2) The disks in solutions at the high pH value appear to be capable of main-

taining a far higher level of potassium than those in solutions at the lower pH values. This is confirmed by the results given in Table II which show the amount of salt which was retained after disks had ceased to absorb salt from solutions at the two pH values after several days. (3) The uptake of chloride was not markedly affected by changes in the pH value of the solution. (4) The uptake from the solution containing buffer at pH 6·1 was very similar to that obtained from the solution without buffer; this suggests that tris had little effect on the uptake of potassium chloride.

TABLE II

Amounts of K^+ and Cl^- (μeq./g.f.w.) held by red beetroot disks at saturation from solutions of 0·0024 M. KCl and 0·024 M. KCl at pH 6·5 and pH 8·5 (temperature = 18° C.). Results are means of five experiments for 0·0024 M. concentration and three experiments for 0·024 M. concentration

		pH	
Concentration	Ion	6·5	8·5
0·0024 M.	K^+	160±17	293±14
	Cl^-	83±14	82±10
0·024 M.	K^+	196±20	294±20
	Cl^-	116±25	86±26

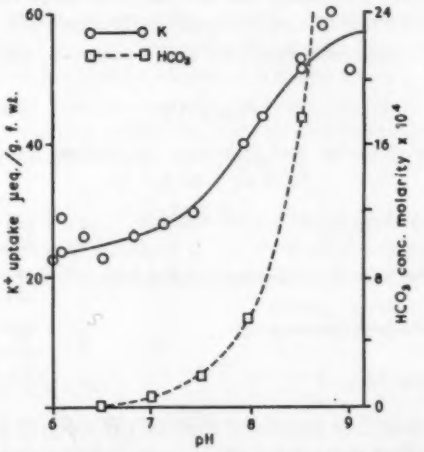

FIG. 2. The influence of pH on (*a*) K^+ absorbed by red beetroot disks in 6 hours at 25° C. from 0·0024 M. KCl solutions, and (*b*) the HCO_3^- concentration in solution obtained by calculation.

The apparent influence of pH shown in these experiments is subsequently referred to as the 'pH effect' on salt uptake. In other experiments conducted over a range of pH values (Fig. 2) it was found that potassium uptake varies directly with pH of the solution between approximately pH 7 and pH 9.

In the course of the investigation it was observed that when the concentration of the salt was increased tenfold, a variation in the pH effect occurred. It was not until approximately 10 to 12 hours after the start of the experiment that any stimulation was observed. The 'pH effect' became more apparent as uptake progressed and the final quantity of salt held by the disks varied with the pH value of the medium in the same way as at the lower concentration (Tables II and III).

TABLE III

Uptake from 0·024 M. KCl in μeq./g.f.w. by beet disks over a 48-hour period at 25° C. Solutions buffered with tris SO_4 at pH 8·5 and 6·5

Time in salt	pH 8·5		pH 6·5		No buffer	
	K^+	Cl^-	K^+	Cl^-	K^+	Cl^-
6 hrs.	71±4	31±3	75±4	35±4	75±3	35±3
24 ,,	164±5	106±4	142±5	102±5	137±6	88±5
48 ,,	227±9	96±4	152±7	102±5	190±8	132±7

Possible explanations of the pH effect. Several different proposals were made and investigated to explain these results. It was thought, for example, that the different treatments might influence the pH of the sap which might control absorption. The pH values of macerates of disks which had been kept in solutions at different pH values for several days were therefore recorded. The values obtained did not vary significantly from pH 5·9 (Table IV) a common value for expressed sap (Fife and Frampton, 1935; Hoagland and Broyer, 1940).

TABLE IV

pH values of macerates from beet disks kept in solutions at different pH values for 6 days at 18° C.

Disks kept in pH 8·5 salt solution	1	pH 5·6
	2	pH 5·9
Disks kept in pH 6·5 salt solution	3	pH 5·9
	4	pH 6·4
Disks kept in water	5	pH 5·9
	6	pH 5·6
Mean value	—	pH 5·9

The possibility was then examined that the pH value of the solution affects the disks in a way that can *subsequently* result in different rates of salt uptake from solutions with an identical pH value. The uptakes of salt at pH 6·5 by disks which had been pretreated for 3 days in buffer solutions without salt at pH 6·5 and 8·5 were, however, found to be identical. This showed that the salt-uptake mechanism was only affected by the pH value of the solution in the presence of the salt.

Another alternative which was considered was that the lower rate of uptake of potassium by disks at the more acid pH might be attributable to an indirect

effect through an increase in the rate of passive leakage. Experiments using disks which had been filled with potassium chloride at pH 6·5 showed that the passive leakage from the cells into buffered salt solutions at 5° C. was very low at both pH values and could not possibly account for the pH effect (Table V).

TABLE V

The potassium chloride lost (μeq./g.f.w.) in 12 hours from filled disks placed in 0·0024 M. KCl solutions at 5° C. unbuffered and buffered with tris sulphate at pH 8·4 and 6·1. Initial concentration in disks: K^+ 264 μeq./g. Cl^- 163 ueq./g.

pH 8·4		pH 6·1		No buffer	
K^+	Cl^-	K^+	Cl^-	K^+	Cl^-
1·5	2·5	4·0	2·5	6·5	4·0

It is also possible that lowering the H^+ ion concentration of the medium increases the amount of cation-carrier complex (RZ) in the cells by increasing the dissociation of the carrier molecule. This might result in a similar increase in the amount of RZ broken down in the cell and hence the amount of cation accumulated. If more cations are accommodated on carrier molecules at higher pH values this might be revealed as an increase in the amount of 'physical' uptake of the tissue at those pH values. The non-metabolic uptake was determined by observing the absorption of potassium chloride in 2 hours by disks at 2° C. At this temperature metabolic uptake is negligible during the uptake period. In fact the uptake observed was the same at both pH values (Table VI). It remains possible that the cations attached to the carrier sites form such a small proportion of the total cations absorbed that changes in their number remain undetected by this technique.

TABLE VI

Uptake of K^+ and Cl^- in 2 hours at 2° C. from KCl solutions buffered with tris SO_4 at pH 8·5 and pH 6·5, and unbuffered at pH 6·5.

Uptake solution	pH	Uptake in μeq./g.	
		K^+	Cl^-
0·0024 M. KCl	8·5	4·0±0·5	0·5±0·1
0·0024 M. KCl	6·5	3·0±0·4	0·5±0·1
0·0024 M. KCl	6·5, no buffer	4·0±0	0·5±0·1

It was during some measurements of the R.Q. of disks in salt solutions at different pH values that it was realized that considerable amounts of bicarbonate were being formed in the more alkaline solutions. The actual amount of bicarbonate in equilibrium with the carbon dioxide in the atmosphere at any pH value is given by the Henderson–Hasselbach equation

$$\mathrm{pH} = \mathrm{K} + \log \frac{[\mathrm{HCO_3}]}{[\mathrm{CO_2}]}.$$

Assuming that there is 0·03 per cent. carbon dioxide in the air, 0·0017 M. bicarbonate is theoretically present in a solution at pH 8·5 in equilibrium with air whilst at pH 6·5 only 0·000017 M. bicarbonate exists in solution at equilibrium (Fig. 2). It may be noted in passing that the cations associated with the bicarbonate in the solutions used here came from the previously undissociated tris molecules of the buffer system. In general, on aerating an alkaline buffered solution the buffer equilibrium is upset and the pH drops slightly from its initial value.

A relationship was observed between the bicarbonate concentration and the cation stimulation at different pH values. Fig. 2 shows that as the pH value was raised above approximately pH 6·5 the stimulation of K^+ uptake and retention of carbon dioxide as bicarbonate in the solution began and followed roughly the same upward curve. This observation suggested that bicarbonate ions were responsible for the pH effect on potassium uptake. If they were taken up by the tissue then extra cations might be accumulated to maintain the electrical balance in the cell. Further evidence to support this hypothesis was obtained when it was found that the uptake of potassium from potassium bicarbonate solutions was greater than from potassium chloride solutions of the same concentration (Table VII). Now if bicarbonate is not absorbed to any extent, as has been sometimes assumed in the past (Hoagland, 1944), one would expect less potassium to be taken up from potassium bicarbonate than from potassium chloride.

TABLE VII

K^+ uptake (μeq./g.f.w.) from 0·0024 M. KCl and 0·0024 M. $KHCO_3$ at 25° C.

Time in hours	KCl	$KHCO_3$
24	20·5	34·0
48	28·0	57·0
72	30·0	66·5
96	30·0	71·5

Now a 0·0024 M. potassium bicarbonate solution has a pH value of about pH 7·6 and it is therefore possible that it was still the pH value rather than the bicarbonate ion which was primarily responsible for the stimulation of cation uptake when the comparison between uptake from bicarbonate and chloride solutions was made. Accordingly the pH value of 0·0024 M. potassium bicarbonate solution was lowered to pH 6·2 by the addition of sulphuric acid. At this pH value theoretically almost all the bicarbonate should have been displaced by the acid, but in fact the acid tip method for estimating bicarbonate released a further volume of carbon dioxide equivalent to 0·0007 M. bicarbonate, demonstrating that the solutions had not reached the theoretical equilibrium and still contained appreciable quantities of bicarbonate ions. Table VIII shows that the potassium uptake from this solution was higher than from potassium chloride although, as might be expected from the lowered bicarbonate concentration, not as high as from potassium bicarbonate solu-

tion at pH 7·6. This suggests that it is not the pH value of the solution *per se* which is stimulating cation uptake, but the presence and probable uptake of bicarbonate ions.

TABLE VIII

K^+ uptake (μeq./g.f.w.) from 0·0024 M. $KHCO_3$ at its 'normal' pH (≃ 7·6) and when reduced to pH 6·2 with H_2SO_4

Uptake period in hours	$KHCO_3$	$KHCO_3$ $+H_2SO_4$	KCl
18	20·5	20·0	10·0
44	39·5	34·5	16·0

Uptake from radioactive bicarbonate solutions. It is more difficult to demonstrate the absorption of bicarbonate than many other salt ions. Neither the simple estimations of the bicarbonate content of the solution nor that in the disks necessarily gives any indication of the amount taken up for the following reasons. Firstly, bicarbonate may be lost from the solution as carbon dioxide by combination with hydrogen ions released from the disks, and, secondly, any bicarbonate accumulated by the tissue may be immediately metabolized to some other compound. By using salts in which the bicarbonate is labelled with ^{14}C both these difficulties can be overcome. However, as has been noted earlier, bicarbonate uptake was usually determined by finding the amount of ^{14}C in the disks and not from the loss of radioactivity of the medium because of complications due to the high rate of exchange in the latter. Under the conditions of the present experiments, in 6 hours at least half the activity in the external solution was found to be lost by this exchange process. (CO_2 free air was passed through these solutions for aeration purposes.) The figures given below for bicarbonate uptake have all been corrected to allow for the loss of external activity by exchange assuming that this loss and the bicarbonate uptake proceed at a linear rate over the course of the experiment. This probably gives a conservative figure for the uptake since the rate of loss of activity will presumably fall off as the overall activity decreases.

Washed disks were pretreated in a solution of inactive 0·0012 M. sodium bicarbonate for an hour (to allow physical uptake of cations to occur) and they were then placed in aerated radioactive sodium bicarbonate solution of the same concentration for 6 hours at 18° C. The tubes were tightly stoppered and the carbon-dioxide-free air passing through the solution was just enough to circulate the disks. The results of two separate experiments in which the uptake of Na^+ and HCO_3^- were determined are quoted in Table IX.

The data show that there was an uptake of bicarbonate almost equivalent to the uptake of sodium ions. The estimates from the radioactivity determinations agreed quite well with those obtained by calculation from the loss of bicarbonate in the external solution (acid-tip method). Subsequent analyses of bicarbonate uptake were made using this second method as it was

considerably more convenient. The uptake values were rather low, partly due to the temperature (18° C.) at which the uptake was performed.

TABLE IX

Amounts of Na^+ and HCO_3^- (μeq./g. f.w.) taken up by red beet disks in 6 hours at 18° C. from 0·0012 M. $NaH^{14}CO_3$ solutions after pretreatment for 1 hour in unlabelled 0·0012 M. $NaHCO_3$. Uptake of HCO_3^- based on (a) *total HCO_3^- in external medium and* (b) *radioactivity measurements on the tissue*

		HCO_3^-	
Replicates	Na^+	(a)	(b)
Experiment A 1	8·3	7·0	9·0
„ „ 2	7·0	6·5	5·5
Experiment B 1	4·5	4·5	5·0

Very little bicarbonate as such was found in the tissue after uptake from potassium bicarbonate solutions; in one experiment, although 69 μeq. of potassium was absorbed in 45 hours and an equivalent amount of bicarbonate lost from solution, only 1·5 μeq. of bicarbonate was found in the tissue. It therefore remained to examine the fate of the accumulated bicarbonate ions.

It has been shown by other workers (Poel, 1953; Graf and Aronoff, 1953, 1955; and Jacobson, 1955) that when CO_2 is fixed from the external solution by plants accumulating salts, the products are mainly organic acids and in particular malic acid. Jacobson and Ordin (1954) have also shown that malic acid is the major acid fixed when excess cations are accumulated. Accordingly it was considered justifiable in the present investigation to confirm the fixation of bicarbonate to malic acid by a relatively crude procedure.

The alcoholic extract from 3·5 g. of disks obtained from an experiment involving uptake from active bicarbonate (as above) was concentrated to a volume of 0·5 ml. A paper chromatogram of this was run using as solvent phenol : water : 90 per cent. formic acid in the quantities 70 g. : 30 ml.: 1 ml. (Stark, Goodban, and Owens, 1951). A streak of the extract was run across the paper at the rate of 14 μl. per 7 cm. Over half the length of this streak an additional streak of 1 per cent. malic acid was pipetted. The enlarged spot on the chromatogram beneath this streak gave the position of malic acid 'corrected' for fluctuations in the Rf. value due to the presence of other substances in the crude extract. The chromatogram was equilibrated for several hours at 18° C. and then run at this temperature. The formic acid was removed at 140° C. (Scott, 1955) and finally sprayed with slightly alkaline bromo-cresol green.

A longitudinal strip was cut from the chromatogram and scanned under an end-window counter. The malic acid spot had by far the highest count (Fig. 3). Other regions which were reasonably active included one on the starting line, presumably a result of adsorption of active molecules on to very slow moving impurities of this relatively crude extract. Some of the other active regions probably correspond with active compounds found by Poel and by Graf and Aronoff (*loc. cit.*); these were not further investigated. The active solutions

were also run in chloroform: ethanol: 90 per cent. formic acid: 6:3:1 (Stark *et al.*, *loc. cit.*) and the malic acid spot confirmed. Other chromatograms of extracts of disks kept in inactive bicarbonate solutions for several days showed that unusually large amounts of both citric and malic acids were present.

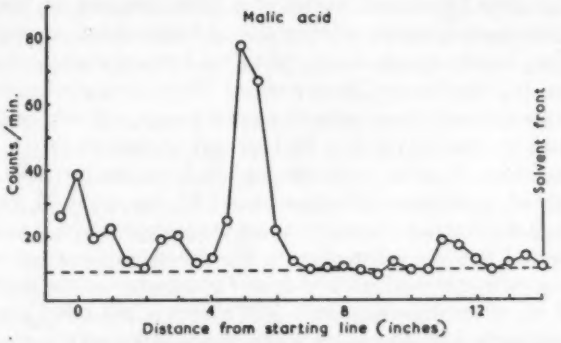

FIG. 3. Solvent: phenol: water: 90% formic acid. 70 : 30 : 1. Distribution of activity along a chromatogram of an extract of beet disks which have been accumulating $NaH^{14}CO_3$.

DISCUSSION

The influence of pH on anion uptake. It has not been possible to test the validity of Jacobson's theory of anion uptake as was originally proposed because of the interference of bicarbonate ions.

These appear in variable quantity in the solution depending on the pH value and the amount of carbon dioxide available. They are taken up by the plant and influence the uptake of the chloride anions making it impossible to isolate any effect on uptake due to the presence of the hydroxyl ions. Nielsen and Overstreet (1955) using excised barley roots have obtained evidence for a competition between bromide and hydroxyl ions by obtaining the uptake of potassium bromide at a range of pH values in unbuffered solutions. They admit that they had difficulty in maintaining the pH value of these solutions and state that their results should only be considered as indicative. Their solutions were aerated and so the presence of bicarbonate ions was inevitable. It seems quite possible that the fall off in bromide uptake that they observed as the pH value of the medium was raised was a result of competition with bicarbonate ions rather than hydroxyl ions.

The 'pH effect' on cation uptake. Considering the magnitude of the effect it is surprising that this has not been observed by other workers. Some have observed relatively slight stimulations in alkaline media, but in no case to the extent reported here.

It might be supposed that beetroot is exceptional in showing this 'pH effect' but it has also been obtained with several other tissues, for example, carrot

and parsnip slices and barley seedlings (Hurd, 1956). Some tissues, however, for example potato and swede storage tissues, do not show the effect.

The results obtained by some other investigators, in which they have compared the uptake of cations from bicarbonate (alkaline) and bromide (neutral) salts are of interest. Ulrich (1941) studied the uptake of potassium salts in 0·005 M. solutions by excised barley roots. He obtained an absorption of potassium from the bicarbonate solution almost double that from the potassium bromide. This discovery was incidental to his investigations and he did not enlarge upon its significance. Jacobson and Ordin (1954) observed similar but less marked stimulations using the same tissue and salt concentration. Hoagland and Broyer (1940) and Nielsen and Overstreet (1955), however, obtained an almost identical potassium uptake from the two salts also using barley roots with a salt concentration of 0·001 M. and 0·005 M. respectively. Thus although the first two observations are in agreement with those presented here, the second two are contradictory. No simple explanation in terms of differences in technique can be found from a comparison of the methods used.

A second set of results which indirectly shows a 'pH effect', comes from workers investigating alkaline chlorosis in plants. Alkaline chlorosis has been shown to be due to the bicarbonate content of the soil and not the inherent alkalinity (Harley and Lindner, 1945). Brown and Wadleigh (1955) working with red beet observed that if sodium bicarbonate was added to the culture solution, considerably greater amounts of cations (largely sodium) were subsequently found in the primary leaves together with a large increase in the organic acid content. The increase in sodium content was not simply due to the addition of more sodium to the culture solution since this made little difference to the overall cation content of the nutrient solution. These results demonstrating exceptionally high cation and organic acid contents are analogous to the experiments described here in which disks of beet are aerated in solutions of potassium chloride for a number of days at different pH values. Brown and Wadleigh have described in effect one of the two manifestations of the 'pH effect' noted in beet disks, namely the very high cation and organic acid level attained in the tissue. It is possible that those plants that readily develop symptoms of alkaline chlorosis exhibit this 'pH effect' on cation uptake whereas those that have some 'resistance' to the disease do not. This possibility has not been examined experimentally.

The increased cation uptake from alkaline solutions has been shown in the present work to be associated with the uptake of bicarbonate ions; it appears that the uptake of bicarbonate serves to increase the total anion absorption and hence also the total cation uptake. This implies that anion uptake is the controlling factor in the uptake process at least in the tissue investigated here, and under the condition of the present experiments; the accumulation of cations occurring as the secondary process essential to the maintenance of electrical neutrality. This does not necessarily mean that anionic accumulation is an 'active' process whilst cation uptake is purely passive. It is equally possible that cationic uptake is an active process but due to the inability of anions to

accumulate rapidly enough it is normally unable to function at its maximum rate. There is the suggestion from experiments in which bicarbonate uptake is estimated in addition to the other salt ions present that total cation and total anion uptakes are always equal; it follows that the rate of uptake of a salt depends on the rate of uptake of the more slowly accumulated ion species.

There appears to be no simple explanation of the higher final level of potassium following uptake from bicarbonate solutions. It seems likely that the explanation will be found when the thermodynamics of the carrier systems have been investigated in more detail. The production of multivalent anions within the tissue means that relatively fewer anion particles are required to balance the cation than if univalent anions are employed indicating that particle number (i.e. an osmotic effect) may be the limiting factor. However, the experimental results obtained do not entirely fit in with this simple idea. Even if all the bicarbonate was converted to trivalent citrate instead of to malate there would still be more salt particles (and therefore a higher osmotic pressure) in the solution in the vacuoles of the cells of the disks filled from a potassium bicarbonate than from potassium chloride solutions.

The uptake of bicarbonate and carbon dioxide fixation. Lundegårdh (1937) concluded that no bicarbonate was taken up by plant roots. He reasoned that this would not occur since the roots were actively excreting carbon dioxide in respiration. In agreement with this conclusion he found that bicarbonate salts had no 'anion respiration'. This latter fact has subsequently been disputed by Handley and Overstreet (1955). Several experiments on beet disk respiration in bicarbonate solutions have been performed in the present investigation. It was obvious that there was a considerable stimulation of respiration in the presence of potassium bicarbonate over the control in distilled water similar to that obtained using potassium chloride solutions (Table X).

TABLE X

Qo_2 of beet disks in KCl, $KHCO_3$ and water at 25° C.

Treatment	Qo_2
0·0024 M. KCl	119±2
0·0024 M. $KHCO_3$	123±2
Dist. water	103±2

Overstreet, Ruben, and Broyer (1940) also concluded that little or no bicarbonate was accumulated by excised barley roots. They used ^{11}C-labelled bicarbonate and since ^{11}C has a half life of only 20·5 minutes they were limited to experimental periods of about 1 hour. They found that a small amount of bicarbonate was fixed but they estimated that 'a several-fold accumulation of potassium over bicarbonate must have taken place'.

It seems likely that in this short period the greater part of the potassium was accumulated by physical exchange processes for hydrogen or other cations in the protoplasm (few anions are absorbed by this form of uptake). Confirmation of this point is difficult because of insufficient details in the original

paper but it appears that the uptake of potassium observed was of the order 5·0 μeq./g.f.w. This is a very reasonable figure for this 'physical' uptake at the concentration of salt they employed (0·001 N.). Hence it is possible that very little potassium was metabolically accumulated and this might be comparable with the low bicarbonate absorption. This experiment appears to be the foundation on which several subsequent references to the absence of bicarbonate uptake are based. The general conception of the role of bicarbonate in salt accumulation is summed up in these words of Hoagland (1944): 'Entering potassium is approximately balanced (in uptake from potassium bicarbonate solution) by newly formed organic acids. If potassium moves in accompanied by bicarbonate ions, the latter are broken down and carbon dioxide is evolved. Bicarbonate ions do not appreciably accumulate in the cell.' Graf and Aronoff (1953, 1955) noted the fixation of carbon from a labelled sodium bicarbonate solution by soya bean and excised onion roots but they were concerned more with the products formed than the method of fixation. Poel (1953) observed the fixation of carbon dioxide supplied to excised barley roots. Jacobson (1955) realized that this fixation was in response to the excess cations taken up from the phosphate buffer that Poel had used. He confirmed this using a range of salts having different cation/anion uptake ratios. The pH value at which both the latter investigations were made was approximately pH 6. Since very little bicarbonate exists in solution at this pH value it is almost certain that carbon dioxide was taken up as such and not as bicarbonate ions. Jacobson obtained a low fixation of external carbon dioxide compared with the increase in organic acids that he observed in the tissue; he supposed that most of the acid was produced from metabolic carbon dioxide. However, there is no doubt that, at least in beet, bicarbonate is fixed much more readily than carbon dioxide; thus in the present experiments although the tissue readily fixes externally supplied bicarbonate it does not utilize its own respired carbon dioxide to a comparable extent. This is the important feature of the 'pH effect' on cation uptake. At pH 6·5 where very little bicarbonate is found in the solution there is only a very slight excess of cations over anions absorbed although if metabolic carbon dioxide were fixed, a considerable excess could be balanced, and uptake at acid and alkaline pH values would not necessarily differ.

There is also considerable evidence that uptake is depressed from solutions through which carbon dioxide is bubbled (Hoagland and Broyer, 1942; Chang and Loomis, 1945). This distinctive reaction to two very similar compounds may be related to the ionic status of the bicarbonate ions as compared with the uncharged carbon dioxide molecules. Bicarbonate is presumably accumulated by the same general mechanism as other charged anions. Carbon dioxide, on the other hand, may well be absorbed by the cell by quite a different mechanism and perhaps indirectly influences ionic uptake through its effects on either respiration or the physical properties of the cell membranes (Kramer, 1940).

The fact that bicarbonate is taken up and fixed by the plant may well lead

to an explanation of other problems in this field especially where the presence of bicarbonate in the uptake solution has been ignored. The work on trace element availability at alkaline pH values and alkaline chlorosis in particular may be assisted by this knowledge. It is possible that at high soil pH values the translocation of trace elements is impeded by the increased rate of accumulation of macroelements, such as potassium, with the result that they are only present in low concentration in the aerial parts of the plant. In support of this, Lindsay and Thorne (1954) using radioactive iron have shown that the direct cause of iron chlorosis at alkaline pH values is the lowered translocation of iron to the leaves rather than a lowered iron uptake by the roots.

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Studies in the Principles of Phytotoxicity

V. INTERRELATIONSHIPS BETWEEN SPECIFIC DIFFERENCES IN SPRAY RETENTION AND SELECTIVE TOXICITY

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SUMMARY

In a detailed study of the factors which control spray retention by the shoot, five species with markedly different morphological characteristics have been examined (*Helianthus annuus*, *Brassica alba*, *Linum usitatissimum*, *Hordeum vulgare*, and *Pisum sativum*). The amount of spray retained, expressed as volume per unit weight of shoot or unit leaf area, is dependent on the stage of development of each species, the volume of spray applied, and the surface tension of the spray solution. The interrelationships between these factors are complex for all the second-order interactions proved to be highly significant. Under one set of conditions there may be a tenfold difference in retention between two species while under another set the corresponding difference may be as little as twofold.

Between a spray output of 14 and 143 ml./m.^{2} the level of retention by *P. sativum* and *H. vulgare* is linearly related to output but for *H. annuus* and *B. alba* the relationship is curvilinear. When the surface tension is reduced below 40 dynes/cm. retention by *P. sativum* and *H. vulgare* is increased, but for *B. alba* and *H. annuus* retention is decreased if the output exceeds 50 ml./m.^{2} For a given output, a reduction in the mean droplet size augments retention by *H. vulgare* but not by *H. annuus*.

Differences in retention either between species or between stages of development are linked with the ratio of leaf area to shoot weight, the nature of the leaf surfaces, the angle of incidence at which the droplets strike the leaf, and localized accumulation. Retention is maximal when the leaf-area ratio is high, the surfaces completely wetted, and the laminae set in a horizontal plane: once this maximum value is reached any additional spray received runs off the shoot.

The relationship between retention and the concentration of the spray solution required to bring about some standard biological response has been examined for two groups of compounds—those which are freely translocated (triethanolamine salts of 2:4-dichloro-, 2:4:5-trichloro-, and 2-methyl-4-chloro-phenoxyacetic acids) and those which are not (ammonium 2:4-dinitro-6-*sec*-butylphenate). For the first group the equi-effective concentration is inversely and linearly related to retention since the total amount received is of primary importance in determining the inhibition of growth, though the position of the droplets must be taken into account. Placing micro-drops containing the growth regulator on the cotyledons of *L. usitatissimum* inhibits the growth of the shoot less than a spray application of the same amount over the whole shoot: for *H. annuus* placing the growth regulator on either the first or second pair of leaves or on the first internode induces more inhibition of growth than application to the cotyledons.

With ammonium dinitro-*sec*-butylphenate the relationship between retention

and concentration is also inverse but approximately logarithmic for *L. usitatissimum*. For some other species the changes in retention consequent upon a reduction in the surface tension are not matched by comparable changes in the equi-effective concentration. For *B. alba* and *H. annuus* though lowering the surface tension may decrease retention it may also diminish the concentration because the fall in retention is more than offset by a greater penetration of the compound into the shoot.

It is concluded that differences in spray retention between species can play a major role in determining selective action and the conditions under which this factor may operate most effectively are discussed.

INTRODUCTION

THIRTY years ago, Aslander (1927) concluded that the effectiveness of dilute sulphuric acid as a selective herbicide for cereal crops was dependent on the differential retention of the spray droplets. Retention was minimal for those species in which an upright habit of growth was combined with a well-developed cuticle and maximal where the leaves were readily wetted and set in a horizontal plane. Subsequently Blackman and Templeman (1936) showed that the addition of a surface active agent to the spray solution could bring about a greater toxicity of the acid to species with a waxy cuticle owing to the enhanced retention. Thus, it was established that retention was dependent both on the morphological characteristics of the shoot and the physical properties of the spray.

The next decade saw an enormous expansion in the development and use of herbicides and with the advent of freely translocated compounds, such as 2:4-dichlorophenoxyacetic acid, the traditional method of spraying a large volume of solution per unit area was in many regions abandoned in favour of a much smaller output per unit area of relatively concentrated solutions. When the present investigation was started in 1951 it was already clear that the phytotoxic effect of a given amount of compound could be markedly influenced by the volume in which it was applied and that this interrelationship changed with the species and the nature of the herbicidal substance (Blackman, 1950). By this time Smith (1946), using a spraying tower, had demonstrated that depressions in the growth of kidney beans induced by 2:4-dichlorophenoxyacetic acid varied with the droplet size and the volume sprayed and that both these factors also influenced retention. Fogg (1948) examining the herbicidal action of dinitro-*o*-cresol concluded that differences in retention between *Sinapis arvensis*, *Avena sativa*, and *Hordeum vulgare* were only partially responsible for the greater susceptibility of *S. arvensis*. Another criterion of importance was the contact angle made between the spray droplet and the leaf surface (Fogg, 1947). If the angle was large, as in cereals, then the area in contact was small and the droplet tended to roll off the leaf. With *S. arvensis*, because of the smaller contact angle, the drops were more stable, and the area of the cuticle through which penetration could take place was larger. Fogg also observed that the contact angle of the cotyledons of *Linum usitatissimum* was smaller than that of the true leaves and that in consequence

differential retention between these parts was likely to arise. Finally, Loomis (1949) had noted that for translocated herbicides coarse sprays of a high surface tension were more effective than a combination of a fine spray and a low surface tension.

While these investigations considered as a whole were indicative of the factors responsible for variations in retention they did not provide a basis for the quantitative assessment of specific differences: nor had they established critically the extent to which the phytotoxic effects were dependent on the retention factor. The present investigation was therefore aimed at a detailed analysis of the extent to which retention is governed by the morphological characteristics of the species, the variations introduced by stage of development, and the changes brought about by altering the spray output or the surface tension of the spray solution. In order to elucidate the relationship between retention and the phytotoxic effects for each level of retention the equi-effective concentration has been determined since only in this way can rigid comparisons be made (Blackman, 1952).

EXPERIMENTAL METHODS

Spraying procedure. The test plants were grown in a mixture consisting of equal parts of sharp sand and sieved soil. Either aluminium or earthenware pots (approximately 25 cm. diameter and 25 cm. deep) were used. The seedlings after emergence were thinned to prevent overcrowding and a complete fertilizer was applied in solution.

The spraying apparatus consisted of a glass bottle fitted with an outlet tube ending in a jet which projected over a bench on which the pots were placed. An air compressor maintained a constant pressure of 2·1 kg./cm.^{2} in the bottle which was mounted on a trolley moving on rails, and was driven by an electric motor and an endless chain past the pots. A spring-loaded tap, held shut by an electromagnet, prevented any premature escape of liquid. At a suitable distance before reaching the first pot the trolley tripped a mercury switch allowing the tap to open and the spray to be emitted.

The output, that is the amount of spray solution applied per unit area, is determined by (*a*) the pressure in the spray container, (*b*) the speed at which the nozzle moves, and (*c*) the size of the orifice in the nozzle. The first two factors were maintained constant (2·1 kg./cm.^{2} and 54 cm./sec.) and the rate of application was varied by using different nozzles. In 1951 two contrasting types of jets with a large and small output were compared, namely a 'Monarch 59' and a 'Bray 00'. In all subsequent experiments a series of 'Teejets' (American Spraying Systems Co.) with a range of orifice sizes was employed. All these nozzles gave a fan-like spray with an angle of 65° in the case of the Teejets.

The final method of calibration adopted was as follows: the jet was placed above the centre of a rectangular metal funnel (25 × 15 cm.) with the long axis of the spray fan parallel to the longer axis of the funnel. The height of the jet above the top of the funnel was adjusted so that it was equal to the height above the top of the pots during spraying. The solution was then passed

through the jet and the spray droplets falling in the funnel for definite periods were collected and the volume measured. The speed of the trolley along the bench was also measured and the output of the jets in ml./m.^{2} calculated.

Estimation of retention. In general, other workers (e.g. Fogg, 1947) have measured retention of the spray droplets by the direct method of weighing the plant before and after spraying. This technique has many drawbacks such as the danger of knocking off spray droplets, evaporation, and the necessity of cutting the plants before spraying with the consequent danger of wilting. Fogg (1948) has also estimated retention of solutions of dinitro-*o*-cresol directly, by washing the plants after spraying and estimating the compound colorimetrically.

In the present investigation, plants were sprayed with a solution of the water-soluble dye, tartrazine. After the spray solution had dried, the shoots were cut at ground level, placed in a suitable stoppered container, and shaken with three or four successive portions of demineralized water and the washings bulked. A little of the wetting agent, 'Teepol', was added to the washing water and the final wash never contained more than a trace of dye. It was established that tartrazine may be recovered quantitatively from a leaf and that there was no detectable loss through penetration. The concentration of tartrazine used was 1 per cent. for jets giving a high output and 5 per cent. for those with a low output. Ordinary laboratory grade tartrazine was used in the initial experiments but with a 6 per cent. solution the surface tension was 62·5 dynes/cm. and in all later experiments tartrazine B.P.C. (supplied by British Drug Houses Ltd.) was employed. The surface tension of a 5 per cent. solution of this material was 68–70 dynes/cm.

The washings from any one replicate were subsequently filtered through glass wool, made up to a known volume and the concentration of tartrazine estimated colorimetrically with a 'Spekker' absorptiometer. From the initial concentration of the dye, the volume of the washings, and the estimated concentration of dye, the volume of solution retained by the plants can be calculated. Using a spectrum violet filter (Ilford 601) which gave the greatest sensitivity for the colorimetric analysis, the final concentration in plant washings had to lie between 0·001 and 0·003 per cent. The minimum volume of washings depended on the bulk of plant material and could be as low as 10 ml. Thus, using a 1 per cent. spray solution, 0·01 ml. of retained solution can be estimated and with 5 per cent. as little as 0·002 ml.

After washing, the plant material was dried in an oven at 98° C. for 24 hours and then weighed. In the early experiments leaf-area measurements were made on a random sample of plants. The leaves were 'blueprinted', and the area of the imprints estimated by planimetry.

Estimation of surface tension. The surface tension of solutions was measured by the standard ring method, using a de Nouy tensiometer. Solutions were made up in 'Permutit' demineralized water, surface tension 71–72 dynes/cm. When the effect of surface tension of the solution was under investigation, solutions were adjusted to the desired value by the addition of a proprietary

surface active agent (Teepol). When wetting agents were used, scrupulous washing of the apparatus was necessary between measurements.

EXPERIMENTAL RESULTS

The interrelationship between the physical characteristics of the spray and retention of the spray droplets by different species.

Preliminary experiments undertaken in 1951, established that for *Pisum sativum* and *Brassica alba* there were specific differences in the degree of retention, and that there was a significant interaction between species and the level of retention resulting from the addition of a surface active agent. It was decided therefore to carry out a comprehensive experiment involving (*a*) five species, (*b*) three spraying occasions at different stages of growth, (*c*) jets giving a high and low output, (*d*) spray solutions with and without the addition of a surface active agent, and threefold replication.

The five species were selected for their contrasting habits of growth and the varying nature of the leaf surfaces. *B. alba* and *Helianthus annuus* represented species with broad horizontal leaves and *Hordeum vulgare* is a plant with upright leaves and a more developed cuticle. *P. sativum* was chosen because of the waxy leaf surface and *L. usitatissimum* as a type where the cotyledons differ from the true narrow leaves in their ease of wetting. The stages of growth on the three spraying occasions are outlined in Table I.

TABLE I

Expt. 1/51. The stages of development of the different species at the time of the spray application

Species	Spraying Occasion I	II	III
Brassica alba	1st 2 leaves expanding; 3rd and 4th just visible. 5–7 cm. high.	3rd and 4th leaves expanding; 5th and 6th just visible. 9–12 cm. high.	5th and 6th leaves expanding; 7th and 8th visible. 12–15 cm. high.
Helianthus annuus	1st leaf pair expanding; 2nd pair 0·5–1 cm. long. 6 cm. high.	2nd pair expanding; 3rd visible. 12–14 cm. high.	3rd pair expanding; 4th visible. 18–24 cm. high.
Pisum sativum	2 leaves expanded; 3rd upright and closed. 5–7 cm. high.	3 leaves expanded; 4th vertical and closed. 8–11 cm. high.	4 leaves open; 5th vertical and closed. 12–15 cm. high.
Linum usitatissimum	8 leaves. 5 cm. high.	12–16 leaves. 9–10 cm. high.	22–26 leaves; cotyledons withering. 17–20 cm. high.
Hordeum vulgare	3 leaves on main axis; 1st tiller just appearing. 15–20 cm. high.	3–4 leaf stage; 20% of plants with one tiller. 25–30 cm. high.	Most plants with one tiller, some with two. 20–30 cm. high.

On each occasion, besides determinations of the dry weight, estimates of leaf area were made, and the mean leaf area, leaf-area ratio, and the dry weight of the shoot are shown in Table II. For the two levels of output a Monarch 59 nozzle, with an output equivalent to 116 ml./m.^{2} ('high volume'), and a

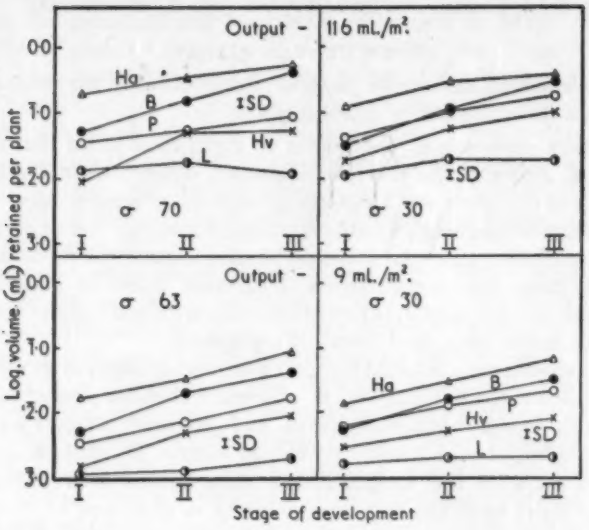

Output - 116 mL./m².
Output - 9 mL./m².
Ha
B
P
Hv
L
1SD
σ 70
σ 30
σ 63
Log. volume (mL) retained per plant
0·0
1·0
2·0
3·0
I
II
III
Stage of development

FIG. 1

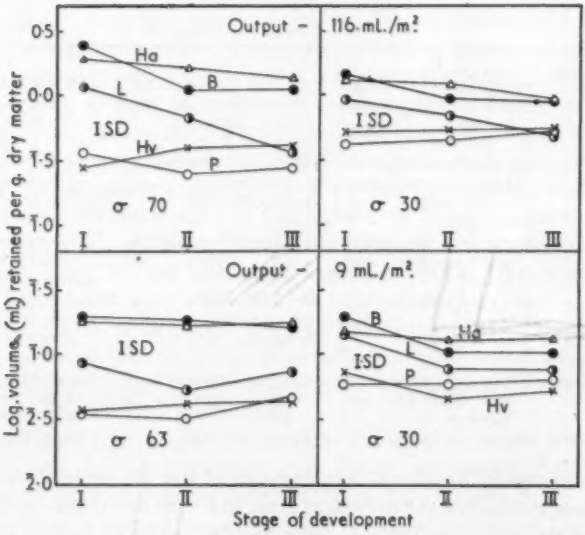

Output - 116 mL./m².
Output - 9 mL./m².
Ha
B
L
P
Hv
1SD
σ 70
σ 30
σ 63
Log. volume (mL) retained per g. dry matter
0·5
0·0
1·5
1·0
2·5
2·0
I
II
III
Stage of development

FIG. 2

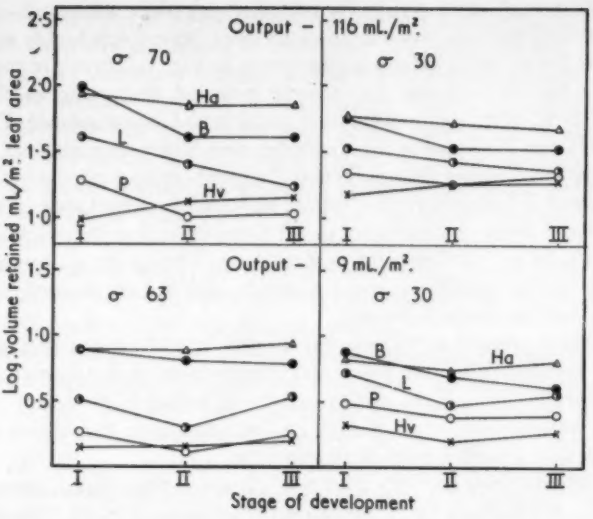


FIG. 3

FIGS. 1, 2, and 3. Expt. 1/51. The inter-related effects of variations in spray output (116 and 9 ml./m.²) and surface tension (σ: 70, 63, and 30 dynes/cm.) on the logarithm of the volume of spray retained by the shoots of *Brassica alba* (B), *Helianthus annuus* (Ha), *Hordeum vulgare* (Hv), *Linum usitatissimum* (L), and *Pisum sativum* (P) at three stages of vegetative development (for details see Tables I and II). Fig. 1. Log volumes retained per plant; Fig. 2. Log volumes retained per g. dry weight; Fig. 3. Log volumes retained per m.² leaf area.

Bray 00 nozzle, with an output of 9 ml./m.² ('low volume'), were used. In this trial ordinary grade tartrazine was employed: for the high output the solution contained 1 per cent. and for the low output 6 per cent., the surface tensions being 69·5 and 62·5 dynes/cm. respectively. When approximately 0·2 per cent. of the surface active agent was added the surface tensions were reduced to 30·4 and 30·1 dynes/cm.

As the variance of the respective data was found to be heterogeneous by Bartlett's test, the data were transformed to a logarithmic basis before carrying out the analyses of variance.

TABLE II

Expt. 1/51. The leaf area and dry weight of the shoots of the different species at the times of the spray application

Species	Leaf area of shoot (cm.²)			Leaf-area ratio (cm.²/g. dry wt. of shoot)			Dry weight of shoot (g.)		
				Spraying occasion					
	I	II	III	I	II	III	I	II	III
B. alba	6·4	36·5	58·1	255	286	262	0·025	0·12	0·30
H. annuus	20·3	47·9	69·7	225	226	198	0·09	0·24	0·45
P. sativum	18·0	53·2	71·7	191	244	253	0·10	0·23	0·35
L. usitatissimum	4·0	7·5	8·4	268	260	209	0·012	0·026	0·040
H. vulgare	14·0	36·8	45·1	339	294	282	0·04	0·11	0·20

When the transformed results for retention per plant were analysed, all the main effects and the first- and second-order interactions were highly significant. The trends for the main effects are apparent in Fig. 1. There are appreciable differences between species, the volume retained decreasing in the order, *H. annuus* > *B. alba* > *P. sativum* > *H. vulgare* > *L. usitatissimum*. With most species retention increases as the shoots become larger, though for *L. usitatissimum* there is no major change. When the nozzle output is low the volume of spray retained is less than from a nozzle with a high output, but it should be noted that the degree of retention is not proportional to the relative outputs of the two nozzles. Lowering the surface tension brings about varying effects depending on the species: in some cases (*B. alba* and *H. annuus*) retention is decreased and in others increased.

Retention expressed as volume per plant is not of great significance from a precise comparative view point and retention on a dry-matter basis is a better criterion. The results of the analysis of variance were very similar to those for retention per plant though in some instances the first-order interactions showed a higher level of significance.

Mean retention per unit dry matter decreases in the order *H. annuus* = *B. alba* > *L. usitatissimum* > *H. vulgare* = *P. sativum* (Fig. 2). Though on this basis the difference between *H. annuus* and *B. alba* and between *H. vulgare* and *P. sativum* are largely eliminated, the difference between the two broad morphological groups is still marked. As might be anticipated retention per g. does not change with age to the same extent as retention per plant. In fact as the plant becomes older there is a tendency for retention to decrease.

The effects of lowering the surface tension depend on the species: for *H. annuus* and *B. alba* retention is decreased, for *P. sativum* and *H. vulgare* it is increased and with *L. usitatissimum* the effect varies both with age and the spray output. The ratios of the volumes retained at high and low surface tensions are set out in Table III and it is clear that with a change to a low

TABLE III

Expt. 1/51. Interrelationships between stage of growth, spray output, and surface tension of the solution on the relative retention of different species

Stage of growth	Spray output (fnl./m.²)	The ratio of retention at a low surface tension to a high surface tension				
		B. alba	*H. annuus*	*P. sativum*	*L. usitatissimum*	*H. vulgare*
Stage I	116	0·58	0·68	1·14	0·82	1·87
	9	1·00	0·83	1·71	1·63	1·85
Stage II	116	0·81	0·73	1·83	1·06	1·37
	9	0·58	0·73	1·88	1·49	1·13
Stage III	116	0·81	0·70	1·92	1·34	1·32
	9	0·68	0·74	1·40	1·05	1·16

surface tension the volume retained is reduced for species which are easily wetted and increased for species which are not.

With a combination of both high output and high surface tension, retention by both *B. alba* and *H. annuus* tends to decrease with age. For *B. alba* the decrease which takes place between the first and second sampling occasions may be accounted for in terms of morphological development. On the first occasion the cotyledons represent a much higher proportion of the total leaf area than on the second sampling date and are more readily wetted than the main leaves. In the presence of a wetting agent the comparable effect of age is rather less evident; under these conditions with a lower surface tension and the consequent better wetting of the surfaces, the spray may run off the leaves or down the stems. When a high surface tension is associated with low output retention changes vary little with stage of development. On the other hand, at a low surface tension there is a decrease with age.

The position for *P. sativum* and *H. vulgare* is more complex. With a high output and a high surface tension, retention by *P. sativum* decreases between the first two occasions but does not change later, whereas with *H. vulgare*, retention at first increases and then does not change. The addition of a wetting agent has a much greater effect at the first occasion for *H. vulgare* but a greater effect at later dates for *P. sativum*. For a low output lowering the surface tension increases retention by *P. sativum* but with *H. vulgare* the effect is small except at the initial sampling. Changes in retention with age are not marked for either species.

The levels of retention by *L. usitatissimum* are intermediate. With a nozzle of high output it is noticeable that in the early stages of growth the volume of solution retained is comparable to that for *B. alba* but when the plants are larger the level of retention resembles more closely that of *P. sativum*. The most likely explanation for this interrelationship, is the change with age in the relative contributions of cotyledons and true leaves to the total leaf surface and to the difference in the two types of surface. On the first sampling occasion only 8 true leaves were present and the cotyledons accounted for 40 per cent. of the total leaf area. On the second date there were 12–16 leaves and the cotyledons contributed 25 per cent. of the total leaf area while on the third date 22–26 true leaves were present and the area of cotyledons was only 16 per cent. of the total.

These proportionate changes in the areas of cotyledons and true leaves would also account for the fact that at a high volume and with a high surface tension, the volume retained per plant is almost independent of age and consequently there is a steady fall in the volume retained per g. At a low surface tension there will be a greater tendency for the spray to run off the cotyledons, but this loss will be offset to some extent by the greater wetting of the true leaves.

For the conditions arising when a nozzle with a low output is substituted the same factors are involved but there is the further effect that retention by the true leaves will be greater because of the smaller droplet size. Droplets are also more likely to lodge in the apical rosette of leaves.

Comparison of Figs. 2 and 3 does not indicate that any marked changes in

the general interrelationships are likely to occur when retention is expressed on a leaf-area basis as opposed to a dry-weight basis. It is evident that the major differences are between species and between conditions of high and low output.

It has already been demonstrated that, according to the species, lowering the surface tension of the spray solution may either increase or decrease the level of retention and it seemed that these relationships warranted further investigations. Accordingly, experiments were carried out in which the two readily wetted species, *B. alba* and *H. annuus*, and two species wetted with difficulty, *H. vulgare* and *P. sativum*, were sprayed using nozzles of different output and with solutions of different surface tensions. The nozzles employed were a series of Teejets (see p. 177) and their outputs covered the range 14 to 143 ml./m.^{2} The solutions of tartrazine (B.P.C.) were adjusted to surface tensions of 70, 50, 40, and 30 dynes/cm.

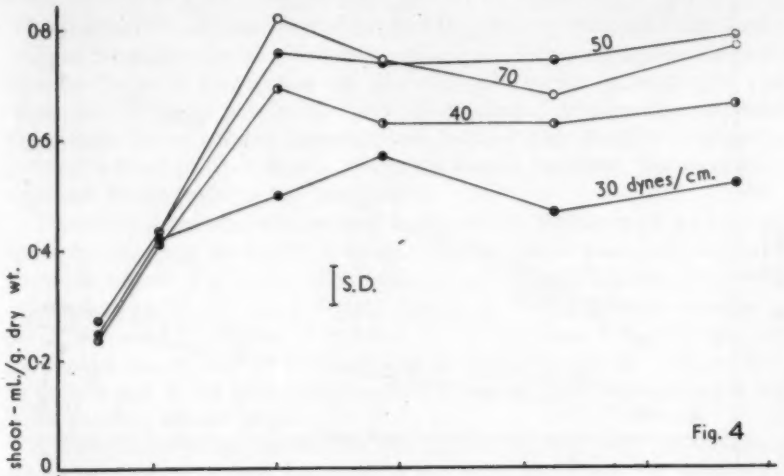
It will be seen from Fig. 4 that between 14 and 50 ml./m.^{2} the degree of retention by *B. alba* increases sharply but that at higher outputs the retention approaches a relatively constant level which is dependent on the surface tension. Save for the lowest surface tension a maximum is reached at 50 ml./m.^{2} but the amount is dependent on the output. At the lowest levels of output the plants are incompletely wetted and it is immaterial whether the drops remain discrete or coalesce. As the output is increased the level of retention can increase so long as the drops remain separate. For a given volume of spray the lower the surface tension the sooner will the drops spread and form a continuous film on the leaves and above a certain thickness the excess solution will run off. Clearly, as the output is increased a film will be formed the thickness of which will be dependent on the contact angle of the solution on the leaf.

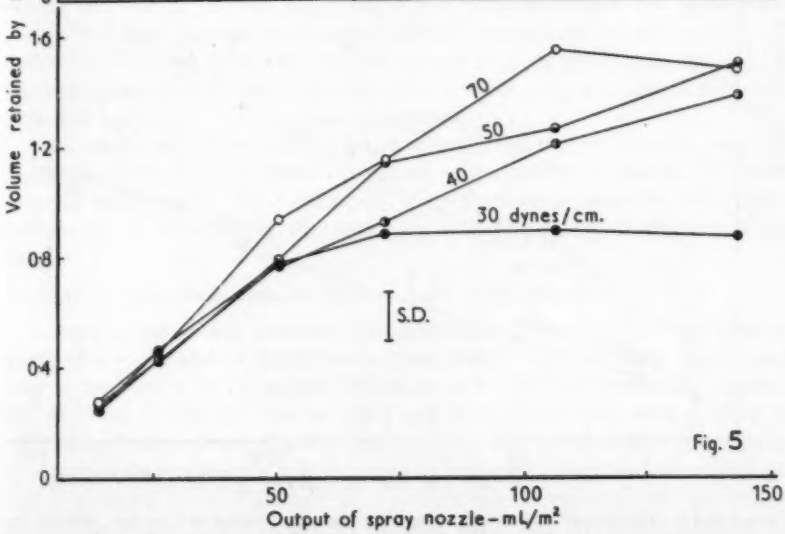
The general trends for *H. annuus* in many respects are similar to those for *B. alba* (see Fig. 5). Between 14 and 50 ml./m.^{2}, irrespective of the surface tension, retention increases, but above 50 ml./m.^{2} the amount and the maximum retained are dependent on surface tension. At the highest tension, retention is maximal at 106 ml./m.^{2} but with solutions of 50 and 40 dynes/cm. retention is still increasing at 143 ml.

The interpretation of these results for 70 and 30 dynes/cm. is similar to that for *B. alba*, namely that retention can increase to a maximum dependent on the contact angle of the solution on the leaves, followed by run-off as the rate of spray application is further increased. At intermediate tensions, the trends for *H. annuus* are rather different due possibly to two opposing trends. As the surface tension is lowered the contact angle of the solution on the leaf is lowered and consequently run-off is facilitated and retention decreases. However, the lower contact angle also enables the droplets to lodge on parts which are more difficult to wet, notably the apical leaves and stem.

For *P. sativum* (Fig. 6) the relationship between retention and spray output is quite different. Firstly, there is no significant difference in retention between tensions of 70, 50, and 40 dynes/cm., but at all outputs there is a very great increase in retention at 30 dynes/cm. Secondly, the degree of retention

is linearly related to output at each surface tension. For the three highest surface tensions all the data can be satisfied by a common regression. It is

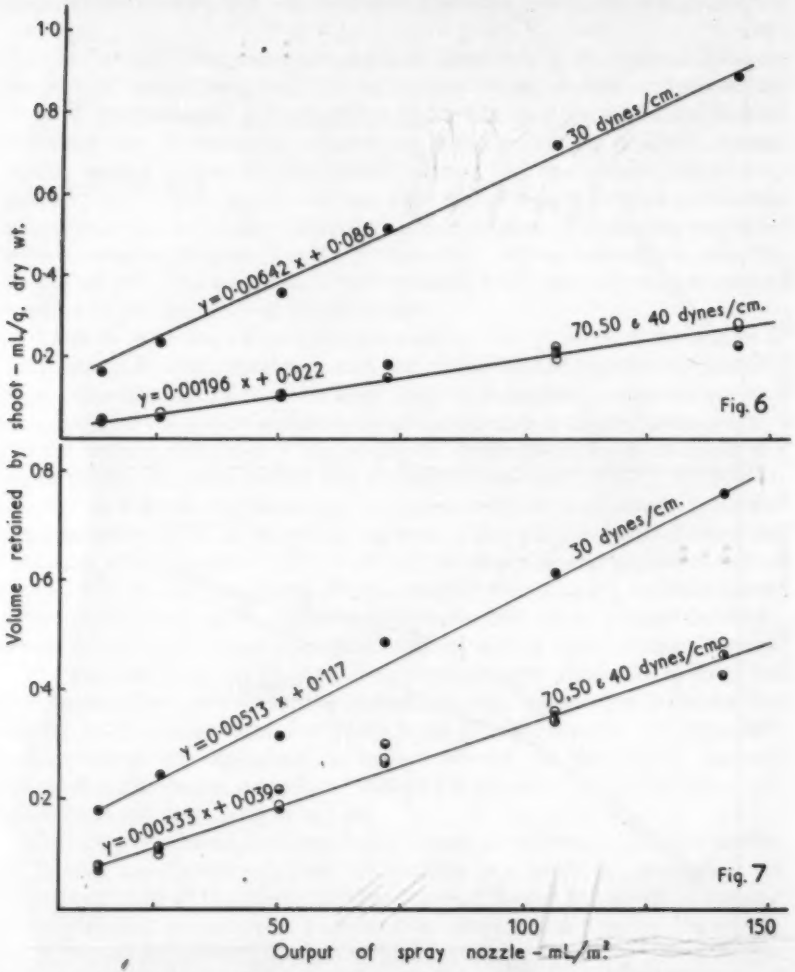




FIGS. 4 and 5. The interrelated effects of variations in spray output and surface tension on the volume of spray retained by the shoot of *Brassica alba* Expt. 1/52 (Fig. 4); the plants were 8 cm. high with the first four leaves of similar size to the cotyledons; and *Helianthus annuus* Expt. 2/52 (Fig. 5); the plants were 15 cm. high and the second pair of true leaves were clearly visible.

not surprising that the addition of a wetting agent should increase retention when the leaves are waxy, but it is of interest that unless the surface tension is

below a critical value, the wetting of the leaves is not greatly enhanced. When the critical value is passed, retention is increased fourfold.



FIGS. 6 and 7. The interrelated effects of variations in spray output and surface tension on the volume of spray retained by the shoot of *Pisum sativum* Expt. 3/52 (Fig. 6); the plants were 12 cm. high with three expanding leaves; and *Hordeum vulgare* Expt. 4/52 (Fig. 7); the plants were 20 cm. high with four leaves on the main axis.

For *H. vulgare* the relationships between retention and volume follow a similar pattern to those for *P. sativum*. Once again (see Fig. 7), retention increases linearly with spray output and there are no major differences between solutions of 70, 50, and 40 dynes/cm. It should also be noted from Figs.

6 and 7 that although the spray output was varied by a factor of 10·4, the comparable figure for the change in retention never exceeded seven. That is to say, the percentage retained falls as the output is increased.

The interrelationships between retention and the frequency of the spray application

In all the experiments so far considered, any alteration in output consequent upon a change in nozzle size has inevitably altered the mean droplet size. Thus, the effects of droplet size and the number of droplets cannot be distinguished. In the present investigation it has not been possible to carry out a detailed study of the influence of droplet size on retention, but an indirect approach to the problem has been made.

The total volume of spray reaching the plants may be increased in two ways, either by changing the nozzle or by spraying the plants more than once with the same nozzle. Fig. 8 gives the results obtained when *H. annuus* is sprayed (*a*) once at 14, 26, 50, 72, 106, and 143 ml./m.^{2} using different nozzles, or (*b*) 1, 2, 3, and 4 times respectively using the same nozzle to give 26, 52, 78, and 104 ml./m.^{2} It is evident that up to *circa* 75 ml./m.^{2}, the variation in droplet size is not important but that at higher volumes retention is less when the droplets are larger.

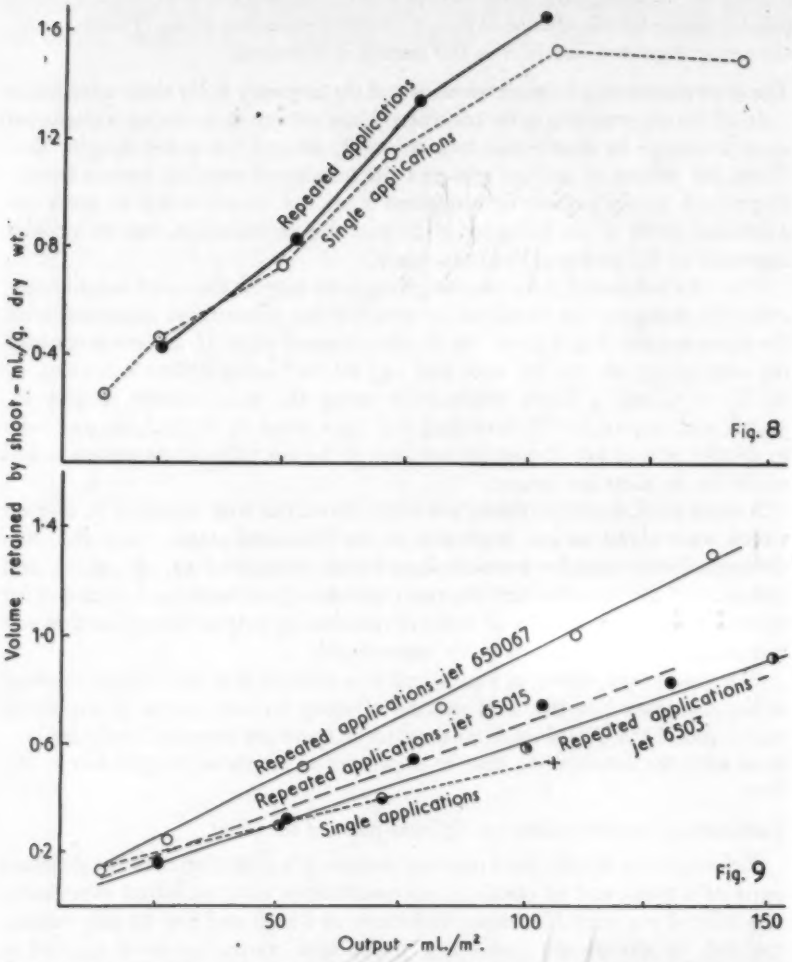
A more extensive experiment was later carried out with plants of *H. vulgare* which were about 20 cm. high and in the three-leaf stage. Altogether five different Teejet nozzles were employed with outputs of 14, 26, 50, 72, and 106 ml./m.^{2} The nozzles with the two highest outputs were used once and for the remaining three nozzles in order of descending output the application was repeated up to 3, 5, and 10 times respectively.

The results are shown in Fig. 9 and it is evident that the volume retained is linearly related to the total volume of spray but the slopes of the linear regressions vary. The slope is steepest when there are repeated spray applications with the smallest jet, that is to say, when the mean droplet size is the least.

Variations in retention between different parts of the plant

Reference has already been made to variations in retention between different parts of a plant and to obtain more quantitative data an initial experiment was carried out with *H. annuus*. Solutions of a high and low surface tension (70 and 30 dynes/cm.) containing 5 per cent. tartrazine were applied at 106 ml./m.^{2} Individual plants before washing were divided into cotyledons, first pair of leaves, second pair of leaves, apical leaves, stem above cotyledons, and hypocotyl. From Table IV it is evident that irrespective of the surface tension the main leaves retain the greatest part. The remaining portions of the plant account for only 16 and 13 per cent. of the total at high and low surface tensions respectively. It is, however, to be noted that lowering the surface tension markedly increases the proportion retained by the apex.

In Expt. 1/51 it was suggested that the complexity of the results for *L. usitatissimum* were in part due to differential retention between the cotyledons and the rest of the shoot. In consequence a more detailed study was made



FIGS. 8 and 9. The comparative effects of repeated applications with a single nozzle and single applications with a range of nozzles on the volume of spray retained by *Helianthus annuus* , Expt. 2/52 (Fig. 8); and *Hordeum vulgare*, Expt. 6/52 (Fig. 9).

involving three stages of growth, two levels each of surface tension and spray output, namely 70 and 30 dynes/cm. and 106 and 26 ml./m.^{2} In addition half the plants had the cotyledons removed. In order to carry out all the spraying on one occasion, batches of pots were sown at intervals which were designed to give suitable differences in the stage of growth. Unfortunately weather conditions were such that at the time of spraying the difference

TABLE IV

Expt. 7/52. Variations in the volume of spray solution retained by different parts of H. annuus

Part of plant	Volume/g. dry weight		Percentage total volume retained	
	High surface tension	Low surface tension	High surface tension	Low surface tension
Cotyledons	0·622	0·454	10·0	8·0
1st pair of leaves	1·726	1·239	39·0	35·0
2nd pair of leaves	2·922	1·578	45·0	34·0
Apex	0·939	2·357	3·8	15·0
Stem	0·118	0·283	1·4	4·9
Hypocotyl	0·084	0·261	0·8	3·1
Whole plant	1·252	0·968	—	—

between the first and second sowings was not very great and the smallest plants were rather more advanced than was intended. For the oldest plants the shoots were 23–28 cm. high, possessed 36–40 leaves, while the cotyledons were yellow or dead. For the medium size the corresponding figures were 15–20 cm. and 30 leaves while the cotyledons exhibited only initial signs of senescense. The smallest plants possessed green cotyledons, 12 leaves, and a height of 7–9 cm.

The cotyledons were cut off at as late a stage as possible before spraying. When washing off the tartrazine the plants with cotyledons were subdivided and in one half the apical whorl of leaves was washed separately.

The data for volume retained per g. dry weight were heterogeneous and have been transformed to logarithms before statistical treatment. The analysis of variance indicated that all the main effects are significant at the 0·001 level and that all the first-order interactions are significant except output × stage of development. Of the second-order interactions only stage of development × surface tension × cotyledons is significant.

From Fig. 10 it is clear, firstly, that the addition of a wetting agent greatly increases retention under all conditions and, secondly, that proportionately the smallest plants retain the most. This size difference is greater at a high surface tension and is more marked when the cotyledons are present. The presence or absence of cotyledons is only of significance at a high surface tension and save for the small plants, only at a high spray output. The 'cotyledon' effects are greatest with the small plants and least with the large ones.

Consideration of the data on the basis of volume retained per plant enables the actual volumes retained by the cotyledons to be assessed by calculating the differences between the mean volumes retained by plants with and without cotyledons. These volumes can in turn be expressed as a percentage of the total volume retained by intact shoots—see Table V. For a combination of a high surface tension and high output the percentage retention by the cotyledons drops with age of plant from 66 to 28 per cent., while for the low output the corresponding figures are 51 and 8·8 per cent. It can also be concluded

that the influence of the cotyledons is not significant when the spray has a low surface tension.

These effects may be explained in terms of the stage of development of the plants and the difference in the characteristics of the true leaves and cotyledons. With the youngest plants the readily wettable cotyledons contribute an

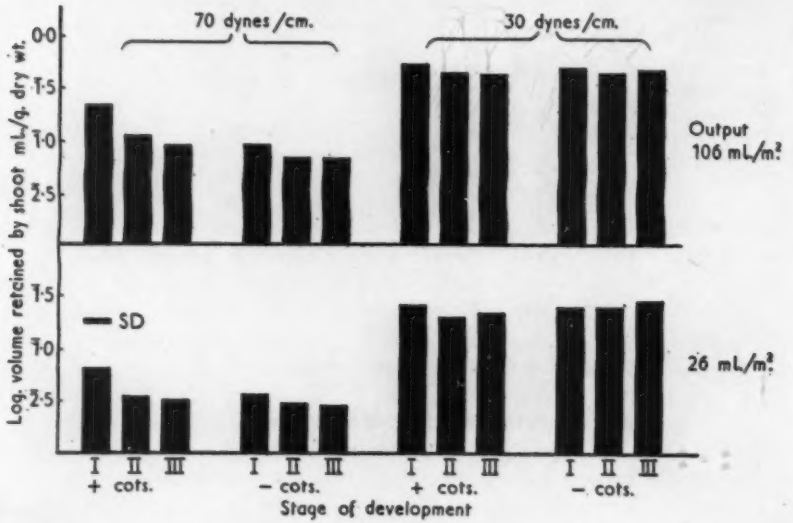


FIG. 10. Expt. 5/52. The interrelated effects of variations in spray output and surface tension on the volume of spray retained by the shoot of *Linum usitatissimum*. Applications were made at three stages of development, on each occasion the cotyledons were removed from half the plants.

important part of the total leaf surface, but at later stages of growth the area of cotyledon relative to the total leaf area decreases and eventually, when the cotyledons have withered, all the retention is by the true leaves. The effect of lowering surface tension is to increase retention by the true leaves and in consequence when the cotyledons are present the gain in retention will be relatively greater for large plants than for small. On the other hand, this increased retention by the true leaves will be partially offset by greater run-off from the cotyledons. These contrasting effects will be most in evidence for small plants and it is not unexpected therefore that at a low surface tension the difference in retention between small plants with and without cotyledons is not significant.

Associated with the combination of low surface tension and low output there will be a fall in the mean droplet size and the increased relative retention could in part be ascribed to a greater tendency of the droplets to lodge in the apical whorls of young leaves. Such a trend is illustrated in Table VI.

TABLE V

Expt. 5/52. Interrelationships between stages of growth, the presence or absence of cotyledons, the spray output and the surface tension of the solution on spray retention by L. usitatissimum

Size of plant	Surface tension dynes/cm.	Spray output (ml./m.²)	Volume retained per 100 plants: With cotyledons (ml.)	Volume retained per 100 plants: Without cotyledons (ml.)	Difference	Difference as % of total	Degree of significance
Small	70	106	0·628	0·211	0·417	66	••
		26	0·179	0·087	0·092	51	•
	30	106	1·52	1·32	0·20	13	—
		26	0·75	0·69	0·06	8	—
Medium	70	106	0·844	0·514	0·330	39	•
		26	0·345	0·244	0·101	29	•
	30	106	4·09	4·00	0·09	2	—
		26	2·09	2·11	−0·02	−1	—
Large	70	106	1·064	0·766	0·298	28	•
		26	0·397	0·362	0·035	8·8	—
	30	106	5·21	6·09	−0·88	−17	—
		26	2·35	2·40	−0·05	−2	—

TABLE VI

Expt. 5/52. The influence of stage of development, the spray output, and the surface tension of the solution on the proportion of the spray retained by apical leaves of L. usitatissimum

Surface tension dynes/cm.	Spray output ml./m.²	Mean volume retained by apical leaves as a percentage of the total: Size of plant: Small	Medium	Large
70	106	9·7	19·1	12·7
	26	12·5	18·2	22·6
30	106	21·7	16·9	13·6
	26	30·5	22·7	24·4

Interrelationships between retention and the concentration of the spray solution required to induce a standard biological response.

*Experiments with ammonium 2:4-dinitro-6-*sec*-butyl-phenate.* So far the experiments have related to the factors controlling retention and no attempt has been made to evaluate the extent to which the amount retained influences the concentration of the compound required to induce some standard phytotoxic effect. To investigate this aspect a number of experiments were first carried out with ammonium 2:4-dinitro-6-*sec*-butyl-phenate where for each combination of nozzle output and surface tension the equi-effective concentration was experimentally determined.

At the same time as the relationships between retention, stage of development, and the removal of the cotyledons from *L. usitatissimum* were being studied (see pp. 188–90, and Fig. 10) comparable batches of pots were sprayed with a range of concentrations and records made of either the percentage mortality of the plants or the degree of damage done to the shoot, based on the

percentage of plants exhibiting severe necrosis. From these data the equi-effective concentrations were determined graphically for each of the combinations of stage of development, presence or absence of cotyledons, high and low surface tension and outputs of 106 and 26 ml./m.[2] In Fig. 11 (*a*) the amount retained has been plotted against log concentration required to kill half the

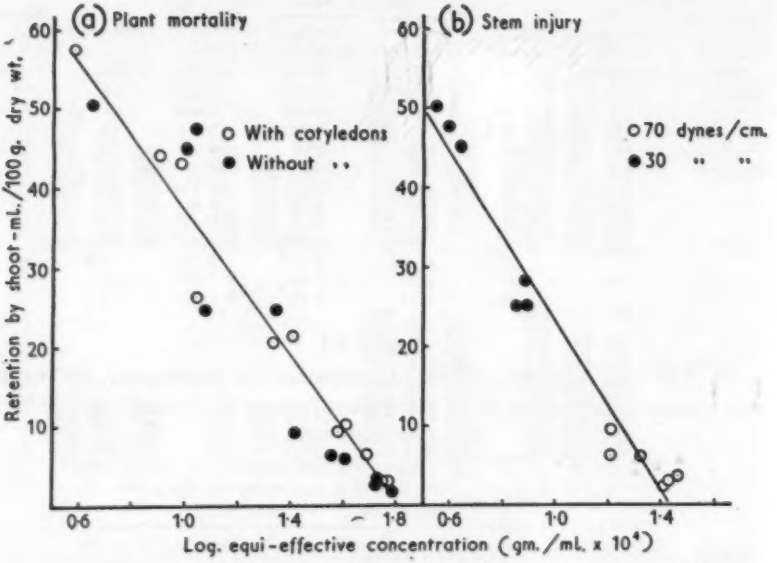


FIG. 11. Expt. 5/52. The interrelationship between the volume of spray retained by the shoot of *Linum usitatissimum* and the logarithm of the equi-effective concentration of ammonium 2:4-dinitro-6-*sec*-butyl-phenate required (*a*) to kill half the plants in the presence and absence of the cotyledons and (*b*) to cause severe stem injury.

plants and it is evident that over all treatments the logarithm of the equi-effective concentration is approximately and inversely proportional to the amount retained per unit weight of the shoot. It is also apparent that the removal of the cotyledons has had no effect on the relationship and this would indicate that the varying amounts retained by the cotyledons (see Table V) do not greatly influence the induced degree of mortality. This assumption is confirmed by the similarity of the equi-effective concentrations in the presence and absence of the cotyledons. For spray applications with a high surface tension the mean difference in concentration is 0·5 per cent. while for a low surface tension a 15 per cent. greater concentration is demanded where there are no cotyledons.

In view of these findings it could therefore be anticipated that a similar logarithmic trend holds for stem injury where the cotyledons have been removed before spraying. From Fig. 11 (*b*) it is seen that this relationship holds

despite the separation of the data into two groups consequent on lowering the surface tension.

In somewhat less extensive experiments with *P. sativum* and *H. vulgare* the equi-effective concentrations for four levels of output and two of surface tension were determined but simultaneous estimates of retention were not undertaken. However, for these species it has been shown that for a given

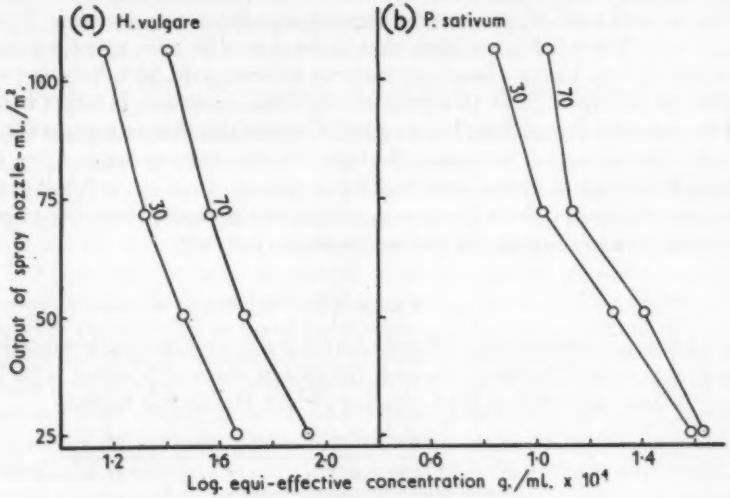


FIG. 12. Expts. 14/52 and 15/52. The effects of variations in spray output and surface tension (70 and 30 dynes/cm., noted on the graphs) on the logarithm of the equi-effective concentration of ammonium 2:4-dinitro-6-*sec*-butyl-phenate required (*a*) to kill half the plants of *Hordeum vulgare* and (*b*) to cause severe injury to the stems of *Pisum sativum*.

surface tension the relationship between output from the spray nozzle and retention can be satisfactorily fitted by a linear regression (Figs. 6 and 7). Therefore, as a measure of retention, output can be substituted and in Fig. 12 output has been plotted against the equi-effective concentration required either to kill half the plants of *H. vulgare* or to cause the collapse of half the stems of *P. sativum*. Within each limit of surface tension the trends are similar to those for *L. usitatissimum*, although the relationship is less clearly linear.

On the basis of the regressions of Fig. 7 for *H. vulgare* a change from a high to a low surface tension increased on average retention by 1·85 while in expt. 1/51 (Table III) the factor ranges from 1·13 to 1·87 with a mean of 1·45. If, therefore, a change in surface tension had no other action than to increase retention there should be a corresponding and counterbalancing reduction in the equi-effective concentrations by 31–46 per cent.: in fact the concentration on average is halved. The discrepancy is clearly not great but it perhaps indicates that with a diminution in the surface tension other factors than a greater retention come into play in causing a larger phytotoxic effect.

From the regressions of Fig. 6 it can be calculated that between outputs of 26–106 ml./m.², decreasing the surface tension from 70 to 30 dynes per cm. increases on average retention of *P. sativum* by 3·4 while for expt. 1/51 (Table III) the ratio is 1·64. The corresponding reductions in the equi-effective concentrations average 23 per cent. as against an expected 39–70 per cent. (Fig. 12 (*b*)). Thus the influence of the surface tension on the level of retention is greater than the effect on the phytotoxicity of the solution.

For *B. alba* and *H. annuus* less direct comparisons can be made. From Fig. IV and Table III it is evident that in the case of *B. alba*, save for a combination of very young plants and outputs below 26 ml./m.², lowering the surface tension from 70 to 30 dynes/cm. depresses retention. It might therefore be expected that at these higher rates of output this lesser retention would lead to a compensatory increase in the equi-effective concentration. From the data of Table VII this trend is evident for an output of 106 ml./m.², but as the output is diminished there is some suggestion of a reversal; lower concentrations are demanded when the surface tension is reduced.

TABLE VII

The interrelationship between surface tension (σ) *and spray output on the concentration of ammonium 2:4-dinitro-6-*sec*-butyl-phenate required to induce a 50 per cent. mortality of either* Brassica alba *or* Helianthus annuus

	Equi-effective concentration—g./ml. × 10^6					
	B. alba				*H. annuus*	
Spray output (ml./m.²)	Expt. 13/53		Expt. 16/53		Expt. 12/52	
σ (dynes/cm.)	70	30	70	30	70	30
106	1·0	1·2	0·6	0·8	3·7	0·9
72	—	—	—	—	6·4	0·9
50	1·2	1·1	1·0	0·8	13·0	1·3
26	2·7	1·7	2·1	1·4	19·0	1·3
13	3·9	3·6	4·2	2·2	—	—

It has already been demonstrated for *H. annuus* that the relation between output and retention varies with the surface tension (Fig. 5). In consequence it is not easy to interpret the trends for the equi-effective concentration given in Table VII. It can, however, be concluded that the reductions in retention induced when the surface tension is diminished over the range of output from 50 to 106 ml./m.² (Fig. 5) do not bring about any complementary increases in the equi-effective concentrations. On the contrary, lesser retention is accompanied by a four to tenfold drop in the equi-effective concentration.

Experiments with chlorinated phenoxyacetic acids. Dinitro-*sec*-butyl-phenol is characteristic of the general class of phytotoxic compounds which are not freely translocated in the shoot and therefore only cause injury to the tissues adjacent to the points of penetration. It seemed therefore of interest to examine the relationship between retention and the physiological effects of readily

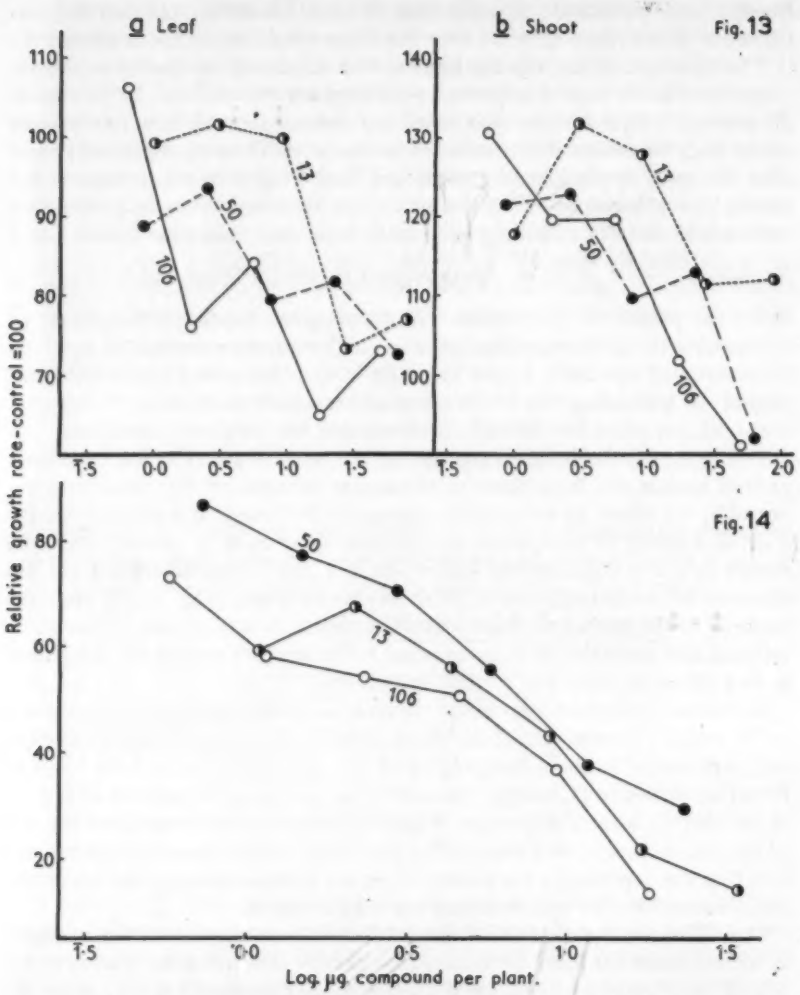
transportable phenoxyacetic acids since it could be anticipated that the distribution of the spray droplets over the shoot would be of less importance.

The spray procedure was the same as that employed for dinitro-*sec*-butylphenol but the criteria of response varied between experiments. In the case of *H. annuus*, which was sprayed when the second pair of true leaves were expanding, the effects were measured in two ways. Over an eight-day period after the spray application the initial and final weights of the laminae of the second pair of leaves and the remainder of the shoot above the first node were determined and the relative growth-rates calculated, using the conventional formula of $\frac{\log_e W_2 - \log_e W_1}{t}$, where W_1 and W_2 are the initial and final weights and t the period of observation. The plants were treated with a range of concentrations of triethanolamine 2:4-dichlorophenoxyacetate for each of three outputs, viz. 106, 50, and 13 ml./m.^{2} Other batches of plants were employed for estimating the levels of retention which were 0·212, 0·134, and 0·044 ml. per plant for the high, medium, and low outputs respectively.

In Fig. 13 (*a*) the induced changes in the relative growth-rates have been plotted against the logarithms of the actual amounts of the compound retained by the shoot. In terms of the changes in leaf weight it is evident that for a given amount of compound the greatest depression is caused when the nozzle output is high and the least when it is low. These differences are less clear-cut when the responses of the shoot are considered (Fig. 13 (*b*)). Here the majority of the treatments have induced a gain in weight. It can, however, be inferred that since the gains are greatest at the smallest output the compound is least effective when it is applied in this way.

A similar experiment was carried out on *L. usitatissimum* and the depressions in the relative growth-rate of the shoot above the first pair of leaves recorded over a period of 12 days. For outputs of 106, 50, and 13 ml./m.^{2} the levels of retention were 0·0057, 0·0034, and 0·0014 ml. per plant. Inspection of Fig. 14 shows that the level of depression is largely dependent on the absolute amount of the phenoxyacetic acid received by the shoot, though there is some suggestion that the depressions are greater when the compound is applied under the combination of a low concentration but a high output.

It will be recalled that when the cotyledons were removed from plants of *L. usitatissimum* the increase in the equi-effective concentration of ammonium 2:4-dinitro-6-*sec*-butyl-phenate did not exceed 15 per cent. (p. 192). A similar experiment was carried out, in which triethanolamine 2:4-dichlorophenoxyacetate was substituted for the nitrophenol. There was, however, an additional treatment. Having determined the level of retention of intact plants when the spray output was 106 ml./m.^{2}, a range of doses were applied by means of an 'Agla' micro-pipette to the cotyledons of a further series of plants. The percentage depressions in the dry weight of the shoot a fortnight after treatment are shown in Fig. 15 (*a*). It should be noted that as the volume of solution applied to the cotyledons was twice the volume retained by the plant, for the purposes of comparison the droplet data have been attributed to a



FIGS. 13 and 14. The influence of variations in spray output on the reductions in the relative growth-rate of (*a*) the second pair of leaves and (*b*) the shoot of *Helianthus annuus* above the first node (Expt. 26/53, Fig. 13), and of the shoot of *Linum usitatissimum* (Expt. 32/53, Fig. 14), induced by the triethanolamine 2:4-dichlorophenoxyacetate deposited on the shoot. Spray outputs in ml./m.^{2} are indicated by the figures on the graphs.

concentration twice that actually applied. It can be concluded, firstly, that removal of the cotyledons markedly increases the concentration required to bring about an equal depression and, secondly, that application to the cotyledons is much less effective than an over-all spray.

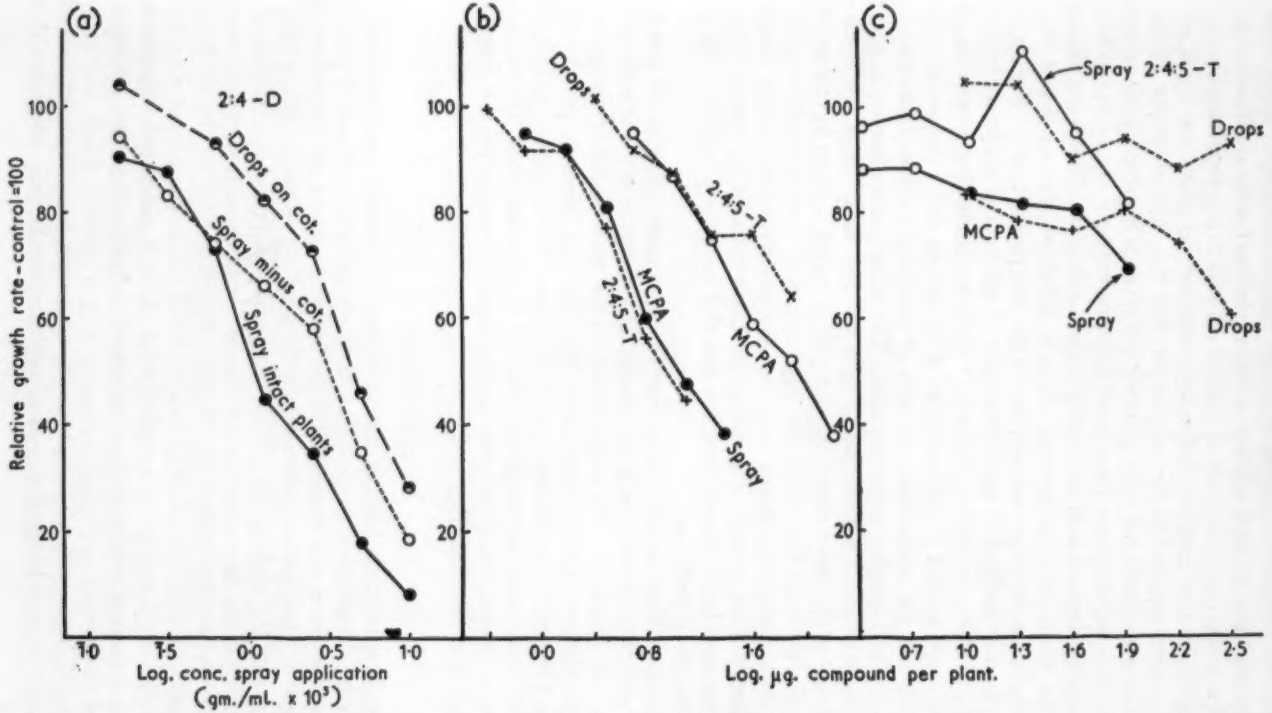


FIG. 15. Expts. 19/53, 22/53, and 21/53. Depressions in the growth-rate of the shoot induced by the triethanolamine salts of (*a*) 2:4-dichlorophenoxyacetic acid and (*b* and *c*) 2-methyl-4-chlorophenoxyacetic and 2:4:5-trichlorophenoxyacetic acids when applied either in an over-all spray (106 ml./m.²) or in micro-droplets to the cotyledons of *Linum usitatissimum* (*a* and *b*) or the first pair of leaves of *Helianthus annuus* (*c*).

In a further experiment, comparisons were made between spray applications at 106 ml./m.^{2} and droplets applied to the cotyledons for the triethanolamine salts of 2-methyl-4-chlorophenoxyacetic acid and 2:4:5-trichlorophenoxyacetic acid. In this instance the volume retained per plant was 0·008 ml. and the volume applied to the cotyledons 0·01 ml. The solutions for the cotyledons contained 2 per cent. glycerol, but no such hygroscopic agent was added to the spray solutions. Once more it is apparent from Fig. 15 (*b*) that for a given dose the depression in the relative growth-rate of the shoot is augmented when the whole shoot is sprayed. Such a difference was not found with *H. annuus* when the drops were applied to the first pair of leaves—Fig. 15 (*c*).

In a further analysis of the importance of differential retention, micro-droplets, totalling in volume 0·04 ml. of solutions containing 2 per cent. of glycerol, were applied to various parts of *H. annuus* and a comparison was made between the triethanolamine salts of 2-methyl-4-chlorophenoxyacetic and 2:4:5-trichlorophenoxyacetic acids. The subsequent changes in shoot growth were recorded in a number of ways but only the effect of the relative growth-rate of the shoot above the first pair of leaves is given in this paper.

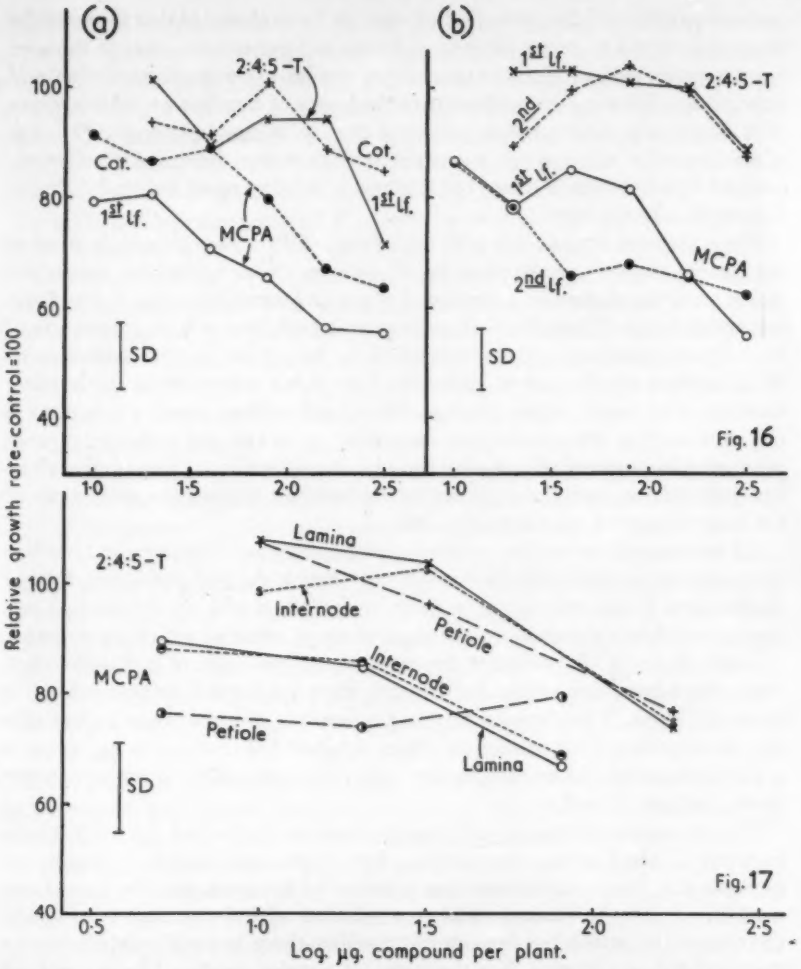
It is evident from Fig. 16 (*a*) that application of the methylchloro-compound to the first pair of leaves, as against the cotyledons, results in a greater inhibition of shoot growth. The difference is less apparent for the trichlorophenoxyacetate but it should be noted that this compound failed in general to depress the growth-rate.

When in another experiment applications were made either to the first or second pair of leaves there were no significant differences in the inhibition of growth—see Fig. 16 (*b*). The next comparison was between micro-droplets on the distal portions of the laminae of the first leaf pair, larger droplets on the junction between the lamina and petiole of the same leaves and droplets placed vertically below each leaf on opposite sides of the first internode. The analysis of variance showed that there were no significant differences for the effect of the point of application (Fig. 17).

DISCUSSION

These investigations have demonstrated that the level of retention can be changed by alterations in spray output, mean droplet size, and surface tension and that the nature and magnitude of the induced changes are dependent on the species. These interactions between the physical properties of the spray solution and the species have been interpreted in terms of the differing morphology of the shoot, particularly the ease or difficulty of wetting the surfaces, the types and position of the leaves and the stage of development. It has been established that for some species retention is only augmented when the surface tension is reduced to a critical value (Figs. 6 and 7) but before considering these results in more detail reference should be made to the findings of other workers.

Ennis, Williamson, and Dorschner (1952) investigating the inhibition of the growth of soya bean by 2:4-dichlorophenoxyacetic acid concluded that



FIGS. 16 and 17. The effects of the triethanolamine salts of 2-methyl-4-chlorophenoxyacetic and 2:4:5-trichlorophenoxyacetic acids on the relative growth-rate of the shoot of *Helianthus annuus* (Expts. 13/53, 14/53) above the first node when applied in micro-drops to (Fig. 16 *a*) the cotyledons or first pair of leaves and (Fig. 16 *b*) the first or second pair of leaves: and (Fig. 17) when applied in micro-drops (i) to the laminae of the first leaf pair, (ii) the junction of the lamina and petiole of the same leaf pair, and (iii) the first internode.

the greater depression induced in a pubescent as against a glabrous variety could be attributed to the greater retention. In a further analysis it was shown that when shoots of both types were inclined at 45 degrees, and exposed to a vertical spray of water or water plus a surface active agent, retention was markedly reduced by approximately the same extent. Employing high-speed

cinematography and droplets of a uniform size it was observed that the majority of aqueous drops bounced off both glabrous and pubescent leaves of the soya bean, but they were retained by some other species, e.g. sugar-beet, *Coleus*, and kidney bean. Even more repellent than the leaves of soya bean were the leaves of cabbage, oats, maize, and a number of grasses. When 1 per cent. of a range of commercial wetting agents was added to the solution the drops were largely retained by all classes of leaves but the type of wetting agent induced different degrees of effectiveness.

More recently Brunskill (1957) has examined in a very thorough manner the factors controlling retention by *P. sativum* under conditions where the leaves were maintained in a horizontal plane and received drops of a uniform but variable size. When the surface tension is high, a very high proportion of large drops (diam. 250–350 μ) bounce off the leaves but below a critical value of the surface tension (45–50 dynes/cm.) there is a sharp rise in the number retained. For small drops (80–95 μ) of a high surface tension retention is almost complete but as the size increases up to 240–325 μ the percentage retained falls progressively. Reducing the surface tension to *circa* 45 dynes/cm. has little influence on the retention of the smallest drops but causes more of the larger drops to remain on the leaf.

All these results were obtained under conditions where the drops had reached their natural terminal velocities when they struck the leaf and it was further shown that if the velocity of a large drop (380 μ and 55 dynes/cm.) was accelerated fivefold then the percentage of drops retained rose from 57 to 93.

Lastly Brunskill investigated the influence of the angle of incidence when large drops impinged on the leaf surface. Here the surface tension was again a critical factor. If the tension was 42·5 dynes/cm., then between a slope of 0 and 60 degrees the percentage of drops retained fell from 95 to 59, whereas at a somewhat higher value of 46·5 dynes/cm. the comparable figures were much lower, namely 38 and 1.

The principles underlying the design of spray nozzles and the mechanisms involved in the formation of droplets have been examined by a number of workers, e.g. Fraser and Eisenklam (1956). For fan-spray nozzles it has been established that the mean droplet size is linked with the surface tension and the area of the orifice but for any given orifice there is a wide distribution in the size of drops formed. For the series of nozzles employed in the present experiments it would seem that the mean droplet size ranged from 100 to 200 μ up to about 300 μ for the largest jet, i.e. a similar range to that investigated by Brunskill.

When the present results for *P. sativum* are considered against the background of Brunskill's findings it would be anticipated that the percentage of the spray retained would fall as the spray output increased (p. 169), since with the associated rise in droplet size a higher proportion would bounce off the shoot. It would also be expected that retention would rise sharply once the surface tension was reduced below a critical level but there is some divergence as to the extent of the reduction required. In Brunskill's experiments marked

changes were observed between 42 and 47 dynes/cm. while from Fig. 6 it is apparent that retention is not greatly augmented until the surface tension is diminished from 40 to 30 dynes/cm. Another discrepancy relates to the effects on retention of a reduction in surface tension at different outputs. On the basis of interaction between droplet size and surface tension recorded by Brunskill reducing the surface tension at a low output should have a smaller effect on the level of retention but this has not been found (Fig. 6).

In making these comparisons it should be emphasized that Brunskill was solely concerned with droplets impinging on leaves while for the shoot of *P. sativum* it has already been pointed out that the spray solution could collect at the base of the petioles. Then again in Brunskill's experiment the leaves were held in a fixed position while in the present experiments the force of the spray, especially at the highest outputs, caused some movement of the shoot.

According to Hellquist (1955) the advancing contact angle for leaves of *P. sativum* is less than that for cereals. It would therefore seem that the fall in percentage retention with increasing output recorded for *H. vulgare* (Fig. 6), can be attributed in part to the failure of the large drops to remain on the leaves. This tendency to bounce off or roll off will be accentuated by the high angle of incidence at which the drops will strike the leaf surfaces.

In the light of the observations of Ennis *et al.* (1952) the data for *B. alba* and *H. annuus* (Figs. 4 and 5) would suggest that the leaf surfaces belong to the class where there is little repulsion of droplets even when the surface tension is high. On such an assumption it would be expected that at a low output diminishing the surface tension would exert little influence on the amount retained. As the output is increased there will come a stage when the droplets just do not coalesce to form continuous films and the retention will be maximal.

From Fig. 8 it can be concluded that for a species like *H. annuus* with a non-repellent leaf surface retention of a drop is largely independent of its size. Unless this was so, retention arising from a single application with a nozzle of high output (up to 72 ml./m.^{2}) and large drops should not equal that due to repeated applications of a jet of low output and smaller drops. In contrast for *H. vulgare* (Fig. 9) a comparable change in spraying procedure brings about appreciable differences in the amount retained. In fact, when the output is within the range of 107–11 ml./m.^{2} retention from a single application compared to a octuple application of a much smaller nozzle may be 45 per cent. less. It is concluded that this reduction is due to the greater repulsion by the leaves of the drops from the single application because of their larger size.

Woofter and Lamb (1953) and Hellquist (1955) have also reported on the relationship between retention and spray output but since in the individual experiments the levels of output were restricted to three or four, precise relationships over a wide range could not be determined. The investigations did, however, demonstrate that, for *Triticum vulgare*, *H. vulgare*, *L. usitatissimum*, *P. sativum*, *Chenopodium album*, and *S. arvensis*, as the output was

increased so the proportion of spray droplets retained diminished. Hellquist also showed that a reduction in the surface tension from 50 to 35 dynes/cm. increased the amounts retained by *P. sativum* and *H. vulgare*.

On the basis of the present results it can generally be concluded that the maximal difference in retention between two species will be attained when the spray consists of large drops with a high surface tension and the leaf surfaces of one species repel the droplets while those of the other do not. From Figs. 2 and 3 it is also apparent that the relative stages of development at the time of spraying should be taken into account. The highest level of retention recorded is for *B. alba* in the early seedling stage when the cotyledons still contribute a considerable proportion of the total leaf surface. For the same conditions of a high output and high surface tension *H. vulgare* retains the least. At later stages of development it is suggested that this difference narrows for several reasons. The leaves of *H. vulgare* no longer hold such an upright position and especially at the leaf tips more spray is retained because the angle of incidence is less and from observation it would seem that the tips are more readily wetted. In contrast, the retention of *B. alba* falls because the true leaves do not collect as much spray as the cotyledons.

From general observation in the field it would seem that for dicotyledonous weeds with epigeal germination the cotyledons are more easily wetted than the true leaves and it follows that where this type of weed is present in a cereal crop from the view-point of the greatest difference in retention early spraying is desirable. Conversely, for dicotyledonous crops it may be advantageous to postpone the application to a later phase, especially if the true leaves of the crop are more repellent than those of the principal weeds. For both sets of conditions it would appear that the volume of spray applied should be so regulated that the amounts retained by the weed species are just below the levels where run off takes place.

So far the discussion has related to specific differences in retention by whole shoots but it is also evident that differential retention by parts of the shoot can take place and that the importance of localized accumulation in determining the over-all phytotoxic effects is dependent on the type of compound. For ammonium dinitro-*sec*-butyl-phenate it has been found that in the case of *L. usitatissimum* the equi-effective concentration is primarily linked with retention by the shoot above the cotyledons (Fig. 11) while for substituted phenoxyacetic acids the amounts retained by the cotyledons contribute to the inhibition of growth (Fig. 15). From Fig. 16 (*a*) it is equally evident that deposits of a growth-regulating compound on the cotyledons induce less inhibition than equivalent amounts on the true leaves, but that at least for *H. annuus* retention by the individual true leaves or the stem produce equivalent effects (Figs. 16 (*b*) and 17).

These investigations have also shown that the part played by retention in determining the equi-effective concentration is dependent both on the species and the compound. For ammonium dinitro-*sec*-butyl-phenate and *L. usitatissimum* it has been found that the expected inverse relation between retention

and the equi-effective concentration is curvilinear and this logarithmic relationship holds whether the retention is altered by varying the output or the surface tension (Fig. 11). Within a given surface tension there is evidence for a similar curvilinear relationship for *H. vulgare* and *P. sativum* (Fig. 12) but the changes in retention due to alterations in the surface tension do not seemingly bring about proportionate changes in the equi-effective concentration. With *P. sativum* the gain in retention due to a decrease in surface tension is not matched by a corresponding fall in the concentration while for *H. vulgare* the position is reversed; in this instance the decrease in the concentration is greater than that expected from the changes in retention (Figs. 6, 7, 12). For *B. alba* and *H. annuus* this trend is accentuated because lowering the surface tension, though it may decrease retention, also diminishes the level of the equi-effective concentration (compare Figs, 2, 4, 5 with Table VII).

These contrasting reactions of the different species could in part be explained if reductions in the surface tension influenced to a varying degree penetration of the nitrophenol into the shoot. On this basis it would be advanced that with *L. usitatissimum* lowering the surface tension primarily effects retention, that for *H. vulgare* the penetration factor comes into operation and that for *H. annuus* the effects on penetration outweigh the variations in retention. So far only preliminary experiments have been undertaken on specific differences in the penetration of nitrophenols. It is apparent from the investigations of Fogg (1947, 1948) that compared to *H. vulgare* there is a more ready penetration of dinitro-*o*-cresol into the leaves of *S. arvensis*. It has been found by one of us (R. S. B.) that if micro-drops containing nitrophenols are placed on the leaves of *H. annuus* then necrotic areas will be produced even if the drops are washed off within 1 to 2 hours. When a wetting agent is added either the drops can be washed off sooner or the concentration required to produce a standard degree of necrosis can be reduced. It is concluded that the wetting agent acts by increasing the area and intimacy of the contact.

The somewhat anomalous results obtained with *P. sativum*, it is considered, are due to differences in the localized retention of spray droplets consequent upon a reduction in the surface tension. At 70 dynes/cm. the spray solution accumulates between the stipules at the junction of the petiole with the stem and causes the stem to be killed or severely damaged. At a low surface tension this accumulation does not take place and though the over-all retention is increased a higher equi-effective concentration is demanded to offset the lack of a localized action.

Turning to the relationship between retention and the activity of substituted phenoxyacetic acids, there is some indication from Figs. 13–17 that with a high output and the associated high retention the growth regulator is slightly more effective but by and large it is the absolute amount deposited on the shoot which determines the order of the inhibition of growth. From this, it follows that the equi-effective concentration required will be inversely proportional to the level of retention and that the concentration will be minimal under conditions where retention is maximal. It also follows that in a

mixed population the optimal selectivity will be attained if the lethal dose for the unwanted species is applied under conditions where the specific differences in retention are maximal. If *B. alba* and *H. annuus* can be taken as representative of some types of dicotyledonous weeds, then from Figs. 4–7 it can be deduced which should be the most effective rate of output to ensure their eradication from *H. vulgare* and *P. sativum*. Considering first spray applications of a high surface tension the most selective output for eliminating *B. alba* from *H. vulgare* is 50 ml./m.^{2} and this conclusion is in general agreement with the previous study of retention of these two species by Holly (1952). With *P. sativum* the same output is applicable for the destruction of *B. alba*. In contrast, a much higher output (106 ml./m.^{2}) is best for the suppression of *H. annuus* in both species. When the surface tension is reduced to 30 dynes/cm. the position is much altered. For *H. annuus* the optimal output is now 50 ml./m.^{2}, for *B. alba* in the presence of *H. vulgare* the figure is the same but changes to 26 ml./m.^{2} when the other component of the population is *P. sativum*.

The reduction in surface tension besides altering the general pattern of the most effective output also changes the specific differences in retention. At the optimal outputs a shift from 70 to 30 dynes/cm. diminishes by 73–76 per cent. the differential retention between *B. alba* and either *H. vulgare* or *P. sativum* while for *H. annuus* the comparable figures are 63–68. Moreover, when the output reaches 106 ml./m.^{2} or more the specific difference in retention can be reversed. For example, more spray is retained by *H. vulgare* and *P. sativum* than by *B. alba*. This reversal is not, however, found in experiment 1/51 (Figs. 2 and 3): at an output of 116 ml./m.^{2}, reducing the surface tension greatly diminishes the specific differences but does not alter the order.

On the basis of these results the generally accepted postulate that in the field the greatest selectivity will be attained at a high output is incorrect. Taking into account the variations due to surface tension it would seem that if *H. annuus* and *B. alba* are morphologically representative of dicotyledenous annual weeds then it would be advantageous to reduce the output to *circa* 50 ml./m.^{2}

It can also be concluded that from the aspect of retention a lowering of the surface tension will bring about a less selective action of a growth regulator. A further important factor will be the influence of the reduced surface tension on the amount penetrating into the shoot. As fort he nitrophenols (p. 203) critical evidence is still lacking on this point. It has been demonstrated that the penetration of sodium 2:4-dichloro-5-iodo-phenoxyacetate into the leaves of *H. annuus* is augmented by the addition of a surface active agent with hygroscopic properties (Blackman and Robertson-Cuninghame, 1955). It has also been found by one of us (K. H.) that the penetration of sodium 2-methyl-4-chlorophenoxyacetate in the leaves of *A. sativa* is accelerated by the inclusion of a wetting agent. There is, however, still much to learn about other species and other compounds.

Finally, while this investigation has aimed at elucidating the part played by differential retention in controlling the selective action of herbicides many

of the results have a bearing on problems associated with the application of fungicides and insecticides, especially where a uniform deposit on the foliage is demanded. The findings also have other implications. At the highest output the conditions are not dissimilar from those of fine rain and differential retention between species may influence the liability to attack by pathogens. It can also be concluded that specific differences in the interception of rain drops may be large enough to be of ecological significance.

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The Translocation of Sulphonamides in Higher Plants

II. ENTRY INTO THE LEAVES OF WHEAT

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SUMMARY

Uptake of a number of sulphonamides and *p*-aminobenzoic and sulphanilic acids can be represented by equations derived from Fick's Law of diffusion. The diffusion constants so derived have a Q_{10} of the order of 1·4 or 1·7, indicating that the process is primarily physical. The Fick's Law equations provide a measure of the permeability of the system and of the size of the effective volume into which the compound is diffusing. Assuming that the basic process is diffusion from water outside the tissue into water inside the tissue, the size of the effective volume is conditioned by the absorption of the compound from the aqueous phase of the free space. With sulphanilamide the effective volume is finite while with *p*-aminobenzoic acid the effective volume is infinite within the limits of the experiment and concentrations up to three times that of the treating solution may be attained. A proportion of the sulphanilamide will diffuse out of shoots immersed in water but *p*-aminobenzoic acid is held irreversibly by the tissue.

INTRODUCTION

IT is well known that chemicals will enter the leaves of higher plants; the success of foliar applications of nutrients, weed-killers, systematic insecticides, and fungicides will depend to some extent on an understanding of the mechanics of penetration. Fogg (1948) attributed the major part of the entry of 3:5-dinitro-*o*-cresol into leaves to diffusion through the epidermis, which was acting as a homogeneous membrane. This process does not appear to have been studied in detail. There are a number of claims, reviewed recently by Kramer (1957), that the initial process by which a chemical enters plant tissue is diffusion. The idea that some part of the plant tissue is freely available for diffusion is implicit in this concept and the study of diffusion tends to centre on this part referred to as the *free space* (Briggs and Robertson, 1957). The nature of the free space precludes its direct measurement and the terms *apparent free space* (Hope and Stevens, 1952; Briggs and Robertson, 1957), *outer space* (Epstein, 1955), or the *diffusion free space* (Johnson and Bonner, 1956) have been applied to estimates of its volume. Some of these estimates are shown in Table I.

Diffusion of the type pictured above is essentially the movement of materials in aqueous solution on the surface of the plant through a membrane into

TABLE I

Estimates of the volume of the apparent free space in various plant tissues (% of tissue volume)

Authority	Tissue	Apparent free space (%)
Hope and Stevens, 1952	Broad-bean roots	13
Butler, 1953	Wheat roots	24·5–33·5
Epstein, 1955	Barley roots	23
Johnson and Bonner, 1956	Oat coleoptiles	19–22
Kylin and Hylmö, 1957	Wheat roots	27·5

water in the free space and the importance of free water or very moist conditions in leaf uptake has been noticed by Weintraub *et al.* (1954) who reported that 2:4-dichlorophenoxyacetic acid was more rapidly absorbed by turgid plants than by plants on the point of wilting; more recently, Zukel *et al.* (1956) have shown that the entry of maleic hydrazide into leaves is markedly dependent on atmospheric moisture. They quote the times required for half the deposit on the leaves to enter the tissue as 128, 25, and 2 hours at relative humidities of 40, 70–75, and 100 per cent. respectively. Conditions of high moisture may actually increase the permeability of the cuticle in addition to increasing the possibility of diffusion of solutes. It appears that the cuticle consists essentially of a hydrophilic framework in which is embedded impermeable wax platelets; in conditions of high humidity the framework will absorb water and swell, this will separate the wax platelets and increase the area available for diffusion (Van Overbeek, 1956).

MATERIALS AND METHODS

A major difficulty in measuring the entry of compounds into plants is assaying the chemical in the tissues. Methods have already been developed for the colorimetric assay of sulphonamides and other primary aromatic amines in plant-tissue extracts and the identity of the compounds assayed has been confirmed on paper chromatograms (Crowdy and Rudd Jones, 1956*a*). These compounds have been chosen for the shoot-uptake studies.

Wheat seedlings (variety, Atlé) were raised on cotton net attached to 3-inch sections of 1·75-inch diameter polythene tube; there were about 20 seedlings on each section; the method was essentially that described by Crowdy, Gardner, Grove, and Pramer (1955). The shoots of 7- or 8-day-old seedlings, which weighed 1 or 2 g., were immersed in 500 ml. of the treating solution for the prescribed periods of uptake. The plants were intact and the roots were kept moist with a wad of damp cotton wool. Unless it is otherwise stated the plants were treated in an incubator at 25° C. After treatment the shoots were washed in running tap water for 5 minutes, harvested and prepared for assay. This washing process has only been found inadequate when plants were treated with high concentrations of sulphadiazine (400μ g./ml.). This compound crystallized on the leaves during immersion, presumably from a super-saturated solution (see Table II). With true solutions no difficulties

have been encountered. In some cases the wad of cotton wool was removed after treatment and the intact plants were returned to nutrient solution and allowed to continue growth; in this way damage due to treatment could be noted. Wheat shoots are difficult to wet and in the earlier experiments 0·1 per cent. Tween 20 was included in the treating solution; later it was found that a pre-treatment of 5 minutes in 0·01 per cent. Tween 20 gave adequate wetting and this pre-treatment was substituted. The presence or absence of wetter in the treating solution did not affect the results (see Table II). Damage due to treatment was immediately apparent as water-soaked areas on the leaves and damaged plants could be eliminated before assay.

The acid dissociation constants quoted were published by Bell and Roblin (1942).

EXPERIMENTAL RESULTS

The general picture presented by the uptake of sulphanilamide by wheat shoots was obtained in a series of preliminary experiments. The effect of Tween 20 as a wetter is illustrated in Table II. Three concentrations of Tween 20 were used, 0·001, 0·01, and 0·1 per cent., and the shoots were immersed in a solution containing 1,000 μg./ml. of sulphanilamide for 5 hours before washing and assay. The wetter was used in two ways: (*a*) The wetter was included in the treating solution for the whole period of treatment. The solutions were not stirred. (*b*) The plants were immersed in solutions of the wetter for 5 minutes before treatment, the wetter was then removed by washing for 5 minutes in running tap water and the plants were treated in the sulphanilamide solution without wetter. The wetter solutions were stirred during the pre-treatment period.

TABLE II

The effect of Tween 20 on the uptake of sulphanilamide by wheat shoots immersed in a solution containing 1,000 μg./ml. Sulphanilamide in shoots is given as μg./g. fresh weight.

Treatment	Tween 20 concentration (%)		
	0·001	0·01	0·1
Continuous treatment	372	567	596
5-min. pre-treatment	565	544	560
Significant difference ($P = 0·05$) must exceed			70
" " ($P = 0·01$) " "			96

The only significant treatment effect is the reduced uptake shown by the continuous treatment with the lowest concentration of wetter; in this treatment it is likely that wetting was not adequate in the absence of agitation.

The amount of sulphanilamide taken up by the immersed shoots varied with the concentration of the solution in which they were immersed and with the period of immersion. When shoots were immersed for 5 hours in solutions containing 200, 300, 400, 500, and 600 μg./ml. of sulphanilamide, the amount assayed in the shoots, s (μg./g. fresh wt.) was linearily related to the

concentration in the treating solution, C (μg./ml.): the relationship was described by the equation $s = 0{\cdot}33C - 12{\cdot}0$. In a second experiment the concentration range was from 100 to 1,000 μg./ml. and the treatment time was 5·5 hours; the same relationship was found to hold and was described by the equation $s = 0{\cdot}31C + 13{\cdot}6$. These lines did not differ significantly in slope and both passed through the zero point within the limits of error. The relationship between uptake and time is considered in greater detail below and is illustrated in Fig. 1. In general the concentration of sulphanilamide in the tissues increased with time of immersion but tended gradually to a maximum.

The tendency for the uptake of sulphanilamide to approach a maximum with time and the linear relationship between uptake and the concentration of the treating solution after a period of immersion could be explained by a model system in which the rate-limiting process is diffusion into the tissues or possibly adsorption to the tissue surface. Both processes may be involved and it may not be possible to distinguish between them. There is, however, some indirect evidence that favours the view that diffusion into the tissues is mainly concerned. Part of this is derived from the experiment detailed in Table II: if surface adsorption were limiting one would have expected competition between the highly surface-active Tween 20 and the sulphanilamide, since none is apparent, diffusion is the more likely process involved. Further evidence that after immersion the compounds have diffused into the tissues is presented in Table III. In these experiments the wheat shoots were inoculated with *Puccinia triticina* Erikss. before and after they had been immersed

TABLE III

The effect of leaf treatment before and after inoculation on the infection of wheat with P. triticina

Compound	Conc. in shoot μg./g. fresh wt.		Time in days from inoculation to			
	Initial	Final	End of trial	Treatment	First symptoms	Final disease rating
Treated prior to infection						
Sulphadiazine	276*	113*	10	0	9	2
	219*	156*	10	0	9	2
	117	94	9	0	No disease	
Sulphacetamide	207	66	10	0	7	1
	82	52	10	0	6	4
Control	—	—	10	—	8	4
Treated after infection						
Sulphadiazine	277	141	14	4	No disease	
	256	191	13	3	No disease	
Sulphacetamide	440	328	14	4	11	1
	328	206	14	4	11	2
	634	273	13	3	11	1
	245	99	13	3	11	2
Control	—	—	14	—	7	4
	—	—	13	—	7	4

* Crystals of sulphadiazine were visible on the leaf surface.

in solutions of sulphadiazine and sulphacetamide. Both these compounds were able to check the development of the pathogen after it had become established in the tissues and this has been taken as evidence that they were present in the same tissues as the fungus in appreciable concentrations. Some indication of the concentration of sulphadiazine in the tissue required to control rust is given by Rudd Jones (1956): the control of *P. coronata* Corda on oats was good with a concentration of 93 μg./ml. in the leaf sap and virtually complete with a concentration of 206 μg./ml. These figures agree well with those presented in Table III and the question of action on the surface does not arise because only the roots of the oat plants were treated. The poor control recorded with free sulphadiazine on the surface of the leaves before inoculation with *P. triticina* is further evidence that this compound has little effect on the surface of the tissues. On the basis of this evidence diffusion into the tissues is considered to be the main process limiting uptake and the following discussion has been based on a diffusion model.

DIFFUSION EQUATIONS

The diffusion of materials into living cells has been considered in detail by Davson and Danielli (1952) and the following is based largely on their account. Basically a diffusion process will follow Fick's Law; the amount of a substance, s, penetrating a thin membrane in a time, t, will depend on the concentration gradient, $C-c$, the area, A, and the permeability constant, k. This can be stated simply

$$\frac{ds}{dt} = kA(C-c). \tag{1}$$

If we assume that the outside reservoir is infinitely large and the inside reservoir has an effective volume, V, then

$$c = \frac{s}{V} \tag{2}$$

and

$$C = \frac{S}{V} \tag{3}$$

where S is the amount of diffusing substance required to raise the concentration inside to a value equal to that outside. Equation (1) may now be expressed in the following form

$$\frac{ds}{dt} = \frac{kA}{V}(S-s). \tag{4}$$

The experimental data to be considered give the total amount of the compound in the tissue after a definite immersion period and this will be expressed by the integrated form of equation (4)

$$e^{-(kA/V)t} = \frac{S-s}{S} \tag{5}$$

or

$$\log_e(S-s) = \log_e S - \frac{kA}{V}t, \tag{6}$$

in this form the data can be fitted with a linear regression and the validity of the model tested. Statistical estimates are also provided of S and kA; V can then be derived from (3). In actual calculation an arbitrary value for S must be selected and the true value obtained by successive approximations. Equation (6) will give valid estimates of S and V but no estimate of A is possible; direct measurement of leaf area would probably only provide an approximate estimate of the surface exposed to diffusion. We have attempted to keep the A factor constant by calculating all figures for a unit weight of shoot tissue: thus, the constant presented below is not the permeability coefficient but is related to it and will be referred to as kA or the permeability constant. The value of kA is unaffected by the units used for measuring the chemical taken up and the unit of time in all cases was 1 hour.

In the following description of the diffusion model *uptake* refers to the total weight (s) of the compound which has entered the tissue in a given time. Uptake is considered as the interaction of two processes which are proceeding simultaneously: *entry*, the diffusion of the compound into an aqueous phase in the free space, and *absorption*, the removal of the compound from this aqueous phase. The rate of entry will be a maximum when the concentration in the aqueous phase is zero. The constant kA provides a measure of the permeability of the external boundaries of the free space and is related to entry. The volume constant, V, referred to as the *effective volume*, does not necessarily measure the size of the free space. The effective volume, as estimated in these experiments, is calculated from the total chemical in the tissue; it will, therefore, be determined by the available water in the free space and by the speed with which the compound is absorbed from this aqueous phase relative to the speed with which it is entering. If no compound is absorbed, the effective volume will have a minimum value equal to the size of the free space; as the rate of absorption increases, the effective volume increases. The actual mechanism of absorption is immaterial.

The rate of absorption from the aqueous phase in the free space can, under uniform conditions, vary from zero to a maximum which equals the maximum rate of entry. When the rate of absorption is less than the maximum, the concentration of the compound within the aqueous phase will increase with time and this increase will be reflected in a decrease in the rate of entry; in these conditions the rate of entry is tending to zero and must, at some time, equal the rate of absorption. At this point the concentration in the aqueous phase within the free space becomes constant and as a result the gradient from the outside to the inside ($C-c$) also becomes a constant, D. Equation (1) now becomes

$$\frac{ds}{dt} = kAD \tag{7}$$

and

$$s = kADt+a. \tag{8}$$

The constant a will be determined by the uptake necessary to reduce the rate of entry to a value equal to the rate of absorption. When the rate of absorption is equal to the maximum rate of entry all the chemical entering the free space

is immediately removed from the aqueous phase with the result that the internal concentration in this phase remains zero and D is equal to the external concentration C. There will have been no initial accumulation and the constant a in equation (8) will also be zero; equation (8) then reduces to

$$s = kACt, \tag{9}$$

a straight line which passes zero. A value for kA can be derived from this equation.

It seems likely that, in the absence of translocation, the absorption curve will also take the general form of equation (1), tending to a maximum. However, as long as the amount absorbed remains small in relation to the absorbing capacity, the rate of absorption will remain substantially constant and linear time uptake curves of the general form of equation (8) will result. Some of the data presented below indicate a very great absorbing capacity within the tissue for some of the compounds examined. Large capacities for absorption have also been recorded for these compounds in the roots of *Vicia faba* (Crowdy and Rudd Jones, 1956*a*).

The technique employed did not permit fine distinctions in the uptake behaviour of the compounds examined, which have been divided into two general classes: *A*, those whose uptake can be fitted statistically to equation (5) in which the value of V is finite; *B*, those whose uptake can be fitted to equation (9) in which the value of V is infinite.

DIFFUSION INTO THE TISSUES

The uptake of sulphanilamide by the shoots at 25° C. is illustrated by a number of experiments summarized in Fig. 1 and Table IV. Fig. 1 shows the agreement between theoretical curves based on Fick's Law and the experimental data for four concentrations of the compound: the agreement is good in each case. The data for the two highest concentrations could also have been fitted with a straight line relating concentration in the tissues to time but this line did not pass zero. The constants kA and V derived from a number of experiments, including those shown in Fig. 1, are presented in Table IV.

TABLE IV

Diffusion of sulphanilamide into wheat shoots at 25° C. Constants derived from diffusion curves

Treatment			Effective volume
Duration hours	Sulphanilamide conc. μg./ml.	kA	V ml./g. (fresh wt.)
7*	1,000	0·07	0·33
16*	500	0·05	0·74
5	500	0·06	0·78
5	500	0·09	0·33
5*	400	0·09	0·63
5	400	0·09	0·61
16*	100	0·07	0·83

* Experiments illustrated in Fig. 1.

This table illustrates the variations which may be expected in kA in a series of experiments; the value was relatively constant over a range of treatment concentrations. Other compounds whose entry into the leaves was fitted with diffusion curves tending to a finite effective volume are listed in Table V: the uptake of sulphadiazine appears to be affected markedly by the pH of the solution used in treatment.

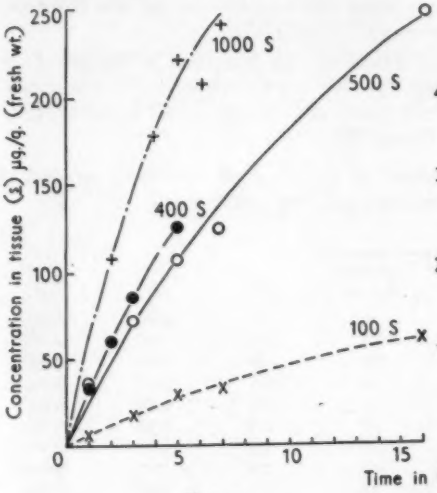

FIG. 1

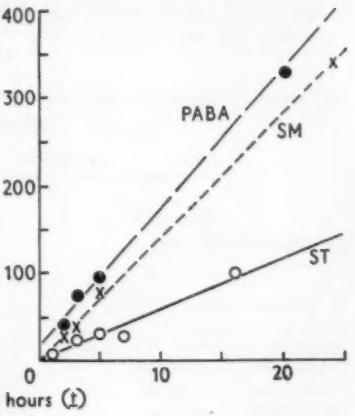

FIG. 2

FIGS. 1 and 2. Diffusion of sulphanilamide (*S*) and *p*-aminobenzoic acid (*PABA*), sulphamezathine (*SM*) and sulphathiazole (*ST*) into wheat shoots. Numbers on graphs are dose rates in µg/ml.

Sulphanilamide	1,000 µg/ml	$e^{-0.21t} = (325-s)/325$
	500	$e^{-0.07t} = (370-s)/370$
	400	$e^{-0.14t} = (254-s)/254$
	100	$e^{-0.08t} = (83-s)/83$
PABA	100	$s = 15.7t+19$
Sulphathiazole	100	$s = 6.0t+0.2$
Sulphamezathine	400	$s = 14.3t-0.03$

TABLE V

Diffusion of chemicals into wheat shoots at 25° C. from a treating solution of 100µg./ml. Constants derived from equation (6)

Chemical	Treatment: Duration hours	pH	pK_a	kA	Effective volume V ml./g. (fresh wt.)
Sulphaguanidine	5	5·1	—	0·04	0·60
Sulphadiazine	5	7·5	6·48	0·04	0·65
Sulphadiazine	16	4·3	6·48	0·08	2·65
4-acetamidobenzenesulphonamide	20	9·5	—	0·07	0·80
4:4′-diaminodiphenylsulphone	16	5·5	—	0·10	1·80
Sulphapyridine	16	5·4	8·43	0·18	0·93

Fig. 2 illustrates the behaviour of compounds for which the effective volume tends to infinity; uptake is linearly related to time and the lines pass through zero. *Para*-aminobenzoic acid was the most striking example of this type of behaviour since shoots immersed in a solution of 100μg./ml. attained an internal concentration of over 300μg./g. (fresh weight) and there was no detectable reduction in rate of uptake at this concentration. Compounds with a low kA may behave as though they were diffusing into an infinite reservoir since the technique will not detect small curvatures.

Compounds whose uptake by the shoots can be described by straight lines passing zero are illustrated in Table VI.

TABLE VI

Diffusion of chemicals into wheat shoots at 25° C. from a treating solution of 100μg./ml. Constants derived from equation (9)

	Treatment			
Chemical	Duration hours	pH	pK_a	kA
p-Aminobenzoic acid	5	—	4·68	0·09
"	20	4·1	—	0·16
"	20	4·0	—	0·12
Sulphamezathine*	24	7·7	7·37	0·04
Sulphacetamide	24	4·2	5·38	0·05
Sulphathiazole*	16	4·8	7·12	0·06
Succinylsulphathiazole	16	6·6	—	0·014
Phthalylsulphathiazole	16	7·0	—	0·015
Sulphanilic acid	16	3·6	3·20	0·02

* Experiments illustrated in Fig. 2.

The effect of temperature on the diffusion of sulphanilamide and *p*-aminobenzoic acid into the tissues is illustrated in Table VII. The uptakes of sulphanilamide at 5° C. and of *p*-aminobenzoic acid at 5° C. and 25° C. were linear functions of time typical of diffusion into an infinite reservoir: at 25° C. the uptake of sulphanilamide tended to a finite effective volume $V = 0{\cdot}33$ ml./g. (fresh weight).

TABLE VII

The effect of temperature on the diffusion of sulphanilamide and p-*aminobenzoic acid into wheat shoots*

	Treatment		kA		
Chemical	Duration hours	Conc. μg./ml.	5° C.	25° C.	Q_{10}
Sulphanilamide	5	500	0·03	0·09	1·7
p-Aminobenzoic acid	20	100	0·06	0·12	1·4

The temperature range of these experiments was chosen to allow a maximum effect on any living processes involved. In calculation it has been assumed that the Q_{10} was constant over the whole temperature range. The measure-

ment of Q_{10} was subject to considerable error and the value was not incompatible with the assumption that the process was primarily physical. In the case of *p*-aminobenzoic acid further evidence that active processes are not involved has been provided by experiments with 2:4-dinitrophenol: a concentration of 10^{-5} M. does no damage to the shoots and has no effect on uptake while 10^{-4} M. causes marked damage and reduces uptake.

DIFFUSION OUT OF THE TISSUES

Compounds which diffuse into the tissues will also be expected to diffuse out when the concentration of the surrounding solution is less than the concentration in the apparent free space. Figs. 3 and 4 illustrate the leaching of

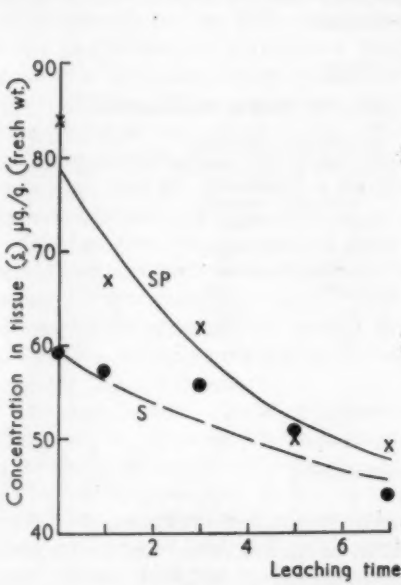

Fig. 3

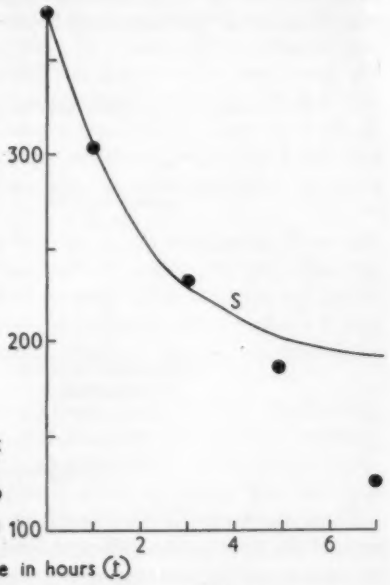

Fig. 4

Figs. 3 and 4. Leaching of sulphanilamide (*S*) and sulphapyridine (*SP*) from wheat shoots.
Fig. 3: *S*, $e^{-0.18t} = (s-30)/30$; *SP*, $e^{-0.24t} = (s-40)/40$.
Fig. 4: *S*, $e^{-0.42t} = (s-188)/190$.

sulphanilamide and sulphapyridine from treated leaves into a large volume of water. In the experiments illustrated in Fig. 3 the compounds were treated initially for 16 hours in a concentration of 100µg./ml. and the leaching curves are adequately described by Fick's Law equations, in each case about half the content of the shoot was freely leachable. In the experiment illustrated in Fig. 4 there was an initial treatment for 16 hours in a concentration of 500µg./ml., in this case the Fick's Law equation could only be fitted for a period of 3 hours, and over this period again about half the sulphanilamide

was freely leachable. Over longer time periods the residue recorded in the shoots fell well below expectation; this, however, might be expected if absorption was a reversible physical process such as diffusion into the cell or partition from the aqueous to a lipoid phase within the free space. There would then be a tendency for the compound to return to the aqueous phase in the free space when the concentration there fell as the result of losses to the outside. If this occurs, diffusion curves can only be fitted while the gradients are low enough to keep the return of the compound to the aqueous phase small in relation to the experimental errors.

Two experiments on the leaching of *p*-aminobenzoic acid are illustrated in Table VIII. There was an initial treatment of 24 hours in a concentration of 100 μg./ml. at 25° C. in the first experiment and for 16 hours at the same concentration in the laboratory in the second.

TABLE VIII

Leaching of p-*aminobenzoic acid from treated wheat shoots*

Leaching time hours	*p*-aminobenzoic acid concentration in tissues μg./g. (fresh wt.)	
	Expt. 1	Expt. 2
0	445	245
1	330*	220
3	348*	202*
5	394	156*
7	456	238
Differences required for significance		
$P = 0{\cdot}05$	66	37
$P = 0{\cdot}01$	92	51

* Significant leaching.

There were significant differences between the *p*-aminobenzoic acid contents of the shoots after different leaching times, but there was no significant regression relating content with time: this acid is leached fairly readily from damaged tissue and some phytotoxic damage may explain the variable results. These experiments indicate a type of binding quite different from that of sulphanilamide and sulphapyridine.

DISCUSSION

The evidence presented above shows that the uptake of these aromatic amines by wheat shoots can be fitted to a simple diffusion model.

The small effect of temperature on the kA values suggests that entry is not an active process and is additional evidence in favour of diffusion entry.

The effect of pH on the uptake of sulphadiazine, illustrated in Table V, is particularly interesting: when the pH of the treating solution is acid the kA value (0·08) is relatively high while in the more alkaline solution the kA value

(0·04) is halved; this might be due to a change in the properties of the diffusing surface but it could also be explained if diffusion depended on the concentration of undissociated sulphadiazine present. Equation (1) shows that the estimate of kA is determined by the estimate of the external concentration, C; at the lower pH sulphadiazine will be virtually undissociated and the concentration of the undissociated molecules in the treating solution will be determined by the weight of the compound dissolved; in these circumstances the value of 0·08 based on this weight may be taken as a true estimate of kA. At the higher pH, on the other hand, the dissociation will be appreciable and the weight of sulphadiazine added to the treating solution will not measure directly the concentration of the undissociated molecules present; an estimate of kA based on the weight of sulphadiazine dissolved in the treating solution will necessarily be low. If the reduction in kA can be attributed to a reduction in the proportion of undissociated molecules, the value of kA obtained is a measure of the concentration of the undissociated molecules present: at the higher pH this will be 50 per cent. of the nominal concentration, from which it can be argued that the pH of the diffusing surface will be about 6·5; this is not an improbable figure. Associated with this revised estimate of kA will be a revised estimate of V, 1·30 ml./g. (fresh weight), which is somewhat closer to the value estimated at the lower pH.

The data give little information about the size of the free space. However, this is the minimum effective volume and will, therefore, be less than the lowest V value recorded, 0·33 ml./g. (fresh weight). This allows a maximum volume for the apparent free space of about 33 per cent. of the tissue, a figure which does not differ greatly from the values, shown in Table I, derived from a variety of plant tissues.

The experiments on leaching indicate two types of absorption. Irreversible absorption, as shown by *p*-aminobenzoic acid, in which the chemical appears to be absorbed completely from the aqueous phase and no leaching occurs, and reversible absorption shown by sulphanilamide, in which the absorbed chemical appears to be in equilibrium with the chemical in the aqueous phase in the free space. Some information on the nature of these types of absorption can be derived from the uptake curves and the effect of temperature on uptake is particularly suggestive. The rate of *p*-aminobenzoic acid uptake is markedly reduced by temperature, but the form of the uptake curve is unaltered since both at 25° C. and at 5° C. the value of V remains infinite and the curve is a straight line described by equation (9); if the rate of absorption had been markedly reduced, a curve of the equation (5) type with a finite value of V would have been expected. Sulphanilamide uptake at 25° C. is of the type described by equation (5) with a finite value of V, while at 5° C. sulphanilamide uptake is a straight line described by equation (9) with an infinite value of V; in this case absorption appears less sensitive to temperature than entry. These experiments lead to the conclusion that an active process plays little part in either type of absorption, a conclusion which is confirmed by the limited experiments with the respiratory inhibitor, 2:4-dinitrophenol. It should be

noted that the behaviour of these two compounds in wheat shoots is comparable with their behaviour in bean roots: *p*-aminobenzoic acid is rapidly taken up by broad-bean roots but is only translocated to a very limited extent while sulphanilamide is less rapidly taken up but is freely mobile (Crowdy and Rudd Jones, 1956*b*).

Any comments on the mechanism of absorption are pure speculation but it seems possible that dissociation is an important factor. It has often been suggested that undissociated molecules enter tissues more readily than ions. It appears that this is true of sulphadiazine and may also apply to *p*-aminobenzoic acid. A high proportion of undissociated molecules of *p*-aminobenzoic acid in the external solution can be ensured by adjusting the pH of the solution; however, within the tissues a pH of the order of 6·5 will be encountered and the acid, which has entered by diffusion of the undissociated molecules, will be almost completely ionized in the free space. The ions may then be adsorbed on to surfaces in the cells and the net effect will be rapid absorption probably irreversible and little affected by temperature. Other compounds which might behave in this way are sulphanilic acid and sulphacetamide. Sulphadiazine, sulphathiazole, and sulphamezathine have pK_a values near the pH of the tissues and at a pH of 6·5 they will be ionized to the extent of 50, 20, and 12 per cent. respectively; they could, therefore, also be subject to the same type of absorption as *p*-aminobenzoic acid. Further, the absorption may be complete if the ions are removed from the aqueous phase since this will drive the equilibrium towards dissociation in the free space. Sulphapyridine will be ionized only to the extent of about 1 per cent. at pH 6·5 and would be less subject to a process of this type. Variation in the uptake behaviour of the compounds in this group may be due to differences in the adsorption of their ions to surfaces in the cells.

The reversible absorption of sulphanilamide (pK_a, 10·3) is unlikely to be an ionization effect and may be a simple process such as partition into a lipoid phase within the cell: a similar process could be postulated for sulphaguanidine, 4-acetamidobenzenesulphonamide, and 4:4′-diaminodiphenylsulphone, none of which are dissociated in aqueous solution. It should be noted that the last compound is most readily soluble in lipoids and also has the highest V value of this group.

It is tempting to compare the uptake of chemicals by wheat shoots with their behaviour when taken up by the roots of broad bean. Very generally, the compounds for which dissociation has been suggested as contributing to absorption are much less mobile in broad bean than those which are virtually undissociated. Sulphaguanidine is an exception to this generalization, but it is poorly taken up by the roots and the shoots. The parallel between absorption in wheat shoots and mobility in broad beans may indicate that only material in the aqueous phase of the free space is available for translocation; but the coincidence may indicate two effects resulting from the same cause rather than cause and effect. Translocation has been described as similar to movement on a chromatogram on which the undissociated molecules are

mobile (Crowdy and Rudd Jones, 1956*b*) and this model is also related to dissociation in the tissues.

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The Translocation of Sulphonamides in Higher Plants

III. ACETYLATION AND DEACETYLATION OF SULPHANILAMIDE IN BROAD BEANS AND WHEAT

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SUMMARY

The acetylation of both amino groups on sulphanilamide has been demonstrated by paper chromatography of sap expressed from broad beans treated with sulphanilamide through the roots. Deacetylation of N^1- and N^4-acetylsulphanilamides occurred in the leaves of treated plants and the N^4-acetyl compound was also acetylated to yield the N^1: N^4-diacetylsulphanilamide.

About 30 per cent. of the sulphanilamide entering broad-bean roots was acetylated in the roots, the maximum proportion of the N^4-acetyl compound being reached after 7 days. Deacetylation is a more limited process but reaches a steady state after the same time. Low levels of acetylation and deacetylation were recorded in wheat.

The amounts of sulphanilamide and N^4-acetylsulphanilamide entering and accumulating in the various tissues were calculated on a water-uptake basis and these data show that the main site of acetylation is in the roots and that deacetylation occurs to a limited extent in the roots but predominates in stems and leaves.

INTRODUCTION

In a preliminary note (Rudd Jones and Wignall, 1955) some account was given of the acetylation of the *p*-amino group of sulphanilamide by broad-bean plants, a process originally recorded in rabbits by Marshal *et al.* (1937). Since then it has been established that deacetylation of the N^4-acetylsulphanilamide can also occur in broad beans.

The present paper describes a quantitative study of acetylation and deacetylation in broad beans and wheat based on chromatographic identification of the compounds involved and measurements of their movement and accumulation in the various tissues. In addition to sulphanilamide (*p*-aminobenzenesulphonamide) and N^4-acetylsulphanilamide (*p*-acetamidobenzenesulphonamide), the N^1-acetyl compound (*p*-aminobenzenesulphonacetamide) and the N^1:N^4-diacetylsulphanilamide (*p*-acetamidobenzenesulphonacetamide) have been included in the study.

EXPERIMENTAL

Broad beans (Sutton's dwarf) were germinated in vermiculite and transferred to nutrient solution in beakers as described in an earlier paper (Crowdy

and Rudd Jones, 1956). There were two plants in each beaker supported by Formica disks on Perspex cylinders. Wheat seed (Atlé), twenty seeds to a beaker, was germinated on a stainless-steel wire mesh (0·022-in. diam. 12 meshes to 1 in.) supported by a Perspex cylinder. The nutrient solution was replaced by solutions containing 200μg./ml. of the compound being studied when the third leaf of the broad bean and the second leaf of the wheat was fully expanded. As with previous experiments the water loss from each beaker was followed and the liquid levels were maintained by making good the loss with treating solution every 3 days and by replacing the solutions each week.

The sampling unit was one beaker. The roots, stems, and leaves of beans and the roots and shoots of wheat were sampled separately. The parts were weighed fresh and killed by immersion in ether for 30 seconds. Excess ether was removed with a filter paper and the sap was expressed from the plant tissue at a pressure of about 400 lb./sq. in. (Crowdy and Pramer, 1955). The ether content of the expressed sap was reduced by evaporation *in vacuo* at laboratory temperature.

A *qualitative* picture of the metabolism of sulphanilamide and its acetyl derivatives was obtained by chromatographic analysis of samples of expressed sap from broad-bean plants which had been treated for 13 or 14 days. The paper chromatograms were developed in *n*-butanol-ammonia-water (4–1–5) and the spots were located by diazotization and coupling either before or after acid hydrolysis (Rudd Jones and Wignall, 1955). Appropriate marker compounds added to the sap of control plants served to identify the chemicals on the chromatogram.

A *quantitative* estimate of the amount of sulphonamide present in the expressed sap was also made using the same reaction employed in identifying the spots on the chromatograms. The method has already been described in detail (Crowdy and Rudd Jones, 1956). The colorimetric test estimates only the primary aromatic amine present and the free sulphanilamide present was determined by adding 0·2 ml. sap to 2·8 ml. water; the solution was then clarified with 15 per cent. trichloracetic acid before diazotizing and coupling. The same test could be used to determine the N^4-acetyl derivatives, since free primary aromatic amines are released by acid hydrolysis; thus a determination made after acid hydrolysis measures the total sulphanilamide content and the N^4-acetyl derivatives will be estimated by the difference between the total and the free sulphanilamide estimations. In estimating the total sulphanilamide content, 0·2 ml. sap was added to 1·4 ml. water, and then clarified with trichloracetic acid: 1·0 ml. N HCl was added and the mixture boiled for 1 hour before neutralizing the acid with 0·4 ml. 2·5 N NaOH and making up to volume with water. Diazotization and coupling were carried out as before.

Samples were taken at varying intervals between 3 and 21 days after the commencement of root treatment.

Localization of the acetylation and deacetylation processes in the tissues

was studied by following the movement of sulphanilamide and N^4-acetylsulphanilamide in cut shoots of broad beans treated through the cut ends, and whole plants treated through the leaves. Shoots of broad bean, cut just above the cotyledon, were placed in 200μg./ml. solutions of sulphanilamide and N^4-acetylsulphanilamide and were sampled at 3, 9, and 13 days after the commencement of treatment. For leaf treatment, shoots were immersed in solutions of the same strength with 0·1 per cent. Tween 20 and 1 per cent. glycerine added. The treating solutions were aerated and the roots were kept moist with damp cotton wool. Shoots were immersed for 5 hours and washed for 5 minutes in running tap water before returning the plants to nutrient solutions. Acetylation and deacetylation were measured as before.

RESULTS

Table I details the compounds identified on paper chromatograms developed from expressed sap of broad beans treated with sulphanilamide and the three acetylsulphanilamides.

TABLE I

The identity and Rf values of sulphanilamide derivatives in sap expressed from broad beans: sulphanilamide (S)*;* N^1*-acetylsulphanilamide* (N^1S)*;* N^4*-acetylsulphanilamide* (N^4S)*; and* N^1:N^4*-diacetylsulphanilamide* (NNS))

Plant part	Treatment	Occurrence and Rf values			
		N^1S	NNS	S	N^4S
Whole plant					
Leaf	S	0·14	—	0·59	0·77
Root	„	0·14	—	0·59	0·77
Leaf	N^4S	—	0·41	0·60	0·78
Root	„	0·14	0·39	—	0·79
Leaf	N^1S	0·13	—	0·57	—
Root	„	0·13	—	—	—
Leaf and root	NNS	——— no spots located ———			
Cut shoot					
Leaf	S	0·13	—	0·59	0·76
Stem	„	0·14	—	0·59	—
Leaf	N^4S	—	—	0·59	0·77
Stem	„	—	—	—	0·77

Sulphanilamide treatment. Acetylation of both amino groups could be detected in root and leaf sap from plants treated with sulphanilamide through the roots. The acetyl compounds may have been present in the leaves as a result of translocation from the roots, but additional evidence from cut shoots indicated that acetylation could take place in stems and leaves.

N^4-acetylsulphanilamide treatment. Free sulphanilamide could be detected in the leaves of whole plants and cut shoots treated with the N^4-acetyl compound showing that deacetylation had occurred. Acetylation of N^4-acetylsulphanilamide had also taken place and N^1-acetylsulphanilamide and N^1:N^4-diacetylsulphanilamide were both identified in expressed sap. The latter compound has only recently been reported from a biological system

(Boyer *et al.*, 1955). The N^1-acetyl compound was only found in the roots of treated plants and the failure to locate it in leaf sap may have been due to poor translocation or to a more active deacetylation mechanism in the leaves. The formation of the N^1-acetyl compound involves hydrolysis in the N^4 position and acetylation in the N^1 position. Neither the N^1-acetyl nor the diacetyl compounds were found in cut shoots and this suggests that they are only formed in detectable amounts in roots.

N^1-acetyl- and N^1:N^4-diacetylsulphanilamide treatments. No acetylation in the N^4 position could be demonstrated in the roots of plants treated with N^1-acetylsulphanilamide, but there was deacetylation in the leaves to yield free sulphanilamide.

In plants treated with the diacetyl compound no spots were located on chromatograms, presumably because there had been insufficient uptake from the treating solution in 14 days.

The proportions of sulphanilamide and the acetyl derivatives resulting from various root treatments are shown as percentages in Table II. When considering this table it must be remembered that the method of assay only distinguishes between substituents in the N^4 position so that N^1-acetylsulphanilamide will be assayed as sulphanilamide and the diacetyl compound as N^4-acetylsulphanilamide. However, since only small quantities of these compounds were present, except in the experiments specifically devoted to them, the results will not be seriously affected.

In plants treated with sulphanilamide the largest proportion of the N^4-acetyl compound was reached after 7 days' continuous treatment and by 18 days there was a fall in the proportion of the acetyl compound in the leaves. This fall was probably caused by a cumulative toxic effect of the treatment resulting in slow hydrolysis in moribund tissue, and it seems likely that a steady state had been reached before this autolytic breakdown intervened. N^4 acetylation of the N^1-acetyl compound took longer to reach the steady state, but after 14 days there was a fall in the proportion of the acetylated compound in the leaves which may have been due to redistribution or to autolysis.

Deacetylation is a more limited process although the steady state was also reached after about 7 days with the N^4-acetyl compound and a rather longer interval for the diacetyl compound. It appears, too, that the deacetylation mechanism operates more strongly in the leaves of broad beans.

There was no clear indication from the root-treatment experiments of any localization of the acetylation process, but the experiments with cut shoots and with plants treated through the leaves (Table III) indicate that the acetyl compound is formed in leaves and stems under these conditions. With leaf treatment, reducing amounts of sulphanilamide and N^4-acetylsulphanilamide were recovered in successive samples. This is due partly to breakdown in the tissues and partly to dilution with growth, but there was no significant difference in the rate of this dilution between the two compounds.

Low levels of acetylation and deacetylation were recorded in wheat by

comparison with broad beans (Table IV) and no free sulphanilamide could be detected in plants treated with N^4-acetylsulphanilamide through the leaves. This suggests that the deacetylation mechanism in wheat is no more active in leaves than in roots.

TABLE II

Acetylated and deacetylated sulphanilamide and N^1*-acetylsulphanilamide in broad beans treated through the roots. Treatments:* S—*200μg./ml. sulphanilamide;* N^1S—*200μg./ml.* N^1*-acetylsulphanilamide;* N^4S—*200μg./ml.* N^4*-acetylsulphanilamide;* NNS—*200μg./ml.* N^1:N^4*-diacetylsulphanilamide*

Time in days	% acetylated						% deacetylated					
	S			N^1S			N^4S			NNS		
	leaf	stem	root	leaf	stem	root	leaf	stem	root	leaf	stem	root
3	19	46	12	0	0	8	10	0	0	0	0	0
7	30	(84)	34	16	0	2	34	0	0	17	0	4
14	29	27	27	6	17	45	16	0	4	28	0	0
18	11	38	31	—	—	—	—	—	—	—	—	—
21	—	—	—	—	—	—	14	7	1	—	—	—

TABLE III

Acetylated and deacetylated sulphanilamide in cut shoots of broad beans (Section I) and broad bean leaves (Section II) treated with 200μg./ml. sulphanilamide (S) *or 200μg./ml.* N^4*-acetylsulphanilamide* (N^4S)

	Time in days	% acetylated S		% deacetylated N^4S	
		leaf	stem	leaf	stem
I	3	27	4	14	7
I	9	34	2	27	4
I	13	19	1	17	0
II	Same day	11		0	
II	5	23		0	
II	8	30		26	
II	13	33		25	

TABLE IV

Acetylated and deacetylated sulphanilamide in wheat treated through the roots with S, *200μg./ml. sulphanilamide, and* N^4S, *200μg./ml.* N^4*-acetylsulphanilamide*

Time in days	% acetylated A		% deacetylated B	
	shoot	root	shoot	root
3	0	0	0	0
6	4	0	2	0
10	4	13	2	0
14	11	20	3	5

The data presented above give no conclusive information on the rates of acetylation and deacetylation or on localization in the tissues. It has already

been shown (Crowdy and Rudd Jones, 1956) that over a period of sulphanilamide uptake by the plant the amount of sulphonamide accumulated in the plant is linearily related to the amount of water transpired; this relationship was again confirmed in the experiments described above, all the regression coefficients presented in Table V were highly significant and in only three cases, indicated in the table, did the lines deviate significantly from linearity; in these cases the deviation was significant at a probability of 0·05 but not significant at a probability of 0·01.

TABLE V

Acetylation of sulphanilamide and deacetylation of N⁴*-acetylsulphanilamide in bean plants treated with 200μg./ml. solutions*

Treatment	Chemical assayed	Rates of accumulation in the tissues μg./ml. water transpired			
		Leaves	Stems	Roots	Whole plant
Sulphanilamide	Sulphanilamide	11·90*	3·23	5·08	20·21
,,	N⁴-acetyl-sulphanilamide	1·71*	1·12	2·40*	5·23
	Total	13·61	4·35	7·48	25·44
N⁴-acetyl-sulphanilamide	Sulphanilamide	1·35	0·13	0·23	1·71
,,	N⁴-acetyl-sulphanilamide	12·05	1·86	6·12	20·03
	Total	13·40	1·99	6·35	21·74

* Regressions deviate significantly from linear at $P = 0{\cdot}05$ but not at $P = 0{\cdot}01$.

The rates presented in Table V are effectively a measure of the concentrations of the compounds present in the transpiration stream at the different stages of translocation: thus the concentration of total sulphanilamide entering the plants treated with sulphanilamide is about 25μg./ml., the concentration entering the stem is about 18μg./ml., and the concentration entering the leaves about 14μg./ml.: part has been accumulated in each organ. The rates of accumulation of sulphanilamide and N⁴-acetylsulphanilamide, based on water uptake, are very similar. There is a marked difference, however, in the rates of accumulation in the stems and roots in the two treatments, the accumulation of the acetyl compound in plants treated with sulphanilamide being about ten times as great as the accumulation of sulphanilamide in plants treated with N⁴-acetylsulphanilamide.

Consideration of the data in Table V suggests that there is a close relation between the concentration of either the sulphanilamide or of its N⁴-acetyl derivative present and the rate of accumulation in the tissue under examination and if a direct proportionality is assumed it is possible to construct flow sheets such as are presented in Tables VI and VII. The figures in these tables were calculated as follows: 25·44μg. of sulphanilamide enter the roots of the plants treated with this compound and as the result of acetylation in the

TABLE VI

Flow sheet for the acetylation of sulphanilamide in broad beans: units are μg. sulphonamide/ml. water transpired

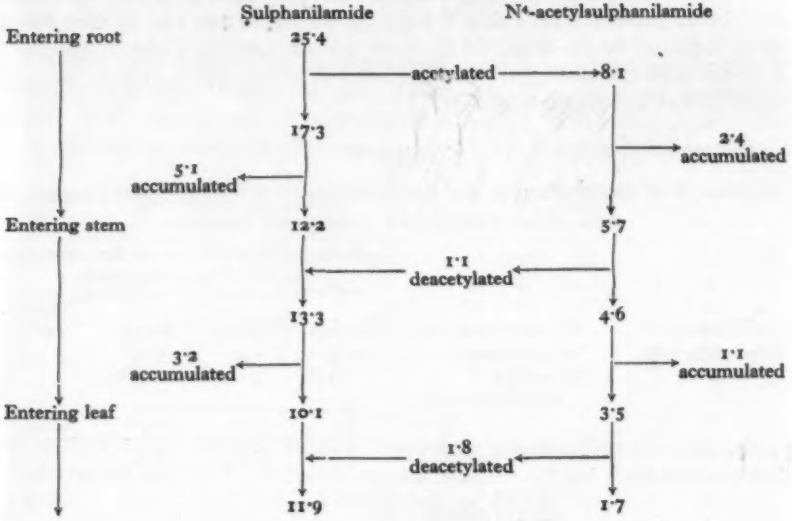
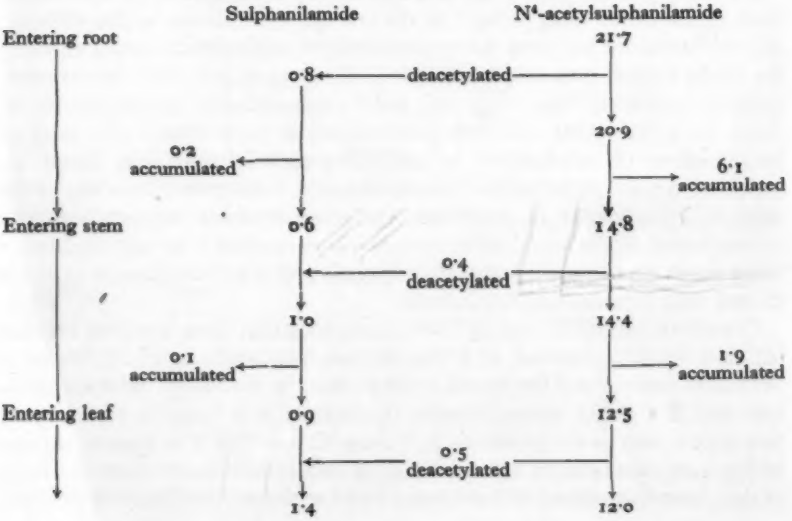


TABLE VII

Flow sheet for the deacetylation of N⁴*-acetylsulphanilamide in broad beans: units are μg. sulphonamide/ml. water transpired*



roots this stream will contain $25{\cdot}44 \times 5{\cdot}08/7{\cdot}48$ (17·28)μg./ml. of sulphanilamide and $25{\cdot}44 \times 2{\cdot}40/7{\cdot}48$ (8·16)μg./ml. of the N^4-acetyl derivative; of these 5·08μg. sulphanilamide and 2·40μg. of the N^4-acetyl will be retained in the roots and the remainder will be transferred to the stem. The amounts received by the stems and leaves and retained in these parts may be calculated in the same way. These figures can only be approximate, but they probably give a dynamic picture of the general process. They show that the main site of acetylation is in the roots in plants treated through the roots, and that deacetylation occurs to a limited extent in roots but predominates in stems and leaves.

DISCUSSION

The evidence from paper chromatogram analysis suggests that sulphanilamide may be acetylated in both the N^1 and N^4 positions in the roots and leaves of broad beans. The results of the cut-shoot and leaf-treatment experiments support this interpretation but analysis of the root-treatment data suggests that if acetylation does occur in the leaves it is masked by the higher level of hydrolysis.

N^4-acetylation of sulphanilamide in the broad bean is a reversible process and the balance between acetylation and deacetylation appears to be determined by the physiological state of the tissues involved. In the roots, the acetylation process predominates and deacetylation occurs only in the presence of a large excess of the N^4-acetyl compound; in general terms the rate of acetylation is about ten times the rate of the reverse process. Both processes occur in the stems but deactylation appears to be favoured. If the leaves are treated directly with sulphanilamide (Table III) the direction of the reaction appears to be determined by the relative concentrations of sulphanilamide and its N^4-acetyl derivative; where the former predominates acetylation occurs and where the latter, hydrolysis. The balance in the leaves, however, is different when the plants are treated via their roots. If the N^4-acetyl derivative is supplied there is a net deacetylation as would be expected but there also appears to be a net deacetylation in the leaves after prolonged root treatment with sulphanilamide. Thus after 18 days' treatment (Table II) the percentage acetylated in the leaves falls to 11 per cent. when about 30 per cent. is acetylated in the stems and roots, in spite of the preponderance of free sulphanilamide present. A similar conclusion is reached in the flow sheets illustrated in Tables VI and VII: these tables suggest that the rate of deacetylation in the leaves is independent of the concentration of the acetyl compound.

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Protein Synthesis in Root Meristems

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SUMMARY

Comparative rates of incorporation of sulphate and L-leucine into proteins have been determined from autoradiographs for the cells of root apices. This has confirmed the existence in most roots of a quiescent centre where the rate of synthesis is much lower than in the surrounding cells. A more detailed account of leucine incorporation in the roots of *Zea* has been made by counting silver grains in quadrats on the autoradiographs. The rate of incorporation per unit volume increases on either side of the quiescent centre and then falls. On the proximal side the lowest rate of incorporation is below that in the quiescent centre and is reached at 1–2 mm. from the tip. Farther from the tip the rate rises. The rate of incorporation per cell does not alter much in the first 2 mm. of the meristem, but beyond that the rate rises steeply to between 15- and 50-fold at 3 mm. from the tip. The patterns of change in rates of incorporation are different for the different tissues of the root.

INTRODUCTION

It has been shown that DNA is synthesized at different rates in the nuclei of different parts of root apices fed with adenine or phosphate (Clowes, 1956*a*, *b*, 1958). In the roots so far investigated all except those with a single totipotent apical cell have a quiescent centre to the meristem where DNA is synthesized at a conspicuously lower rate than in the surrounding tissues. The quiescent centre is a roughly hemispherical region at the apex of the stele and cortex. It extends across about half or a third of the width of the apex and contains about 500 cells in *Zea mais*, 600 cells in *Sinapis alba*, and 1,100 cells in *Vicia faba* primary roots. The low rate of DNA synthesis in the quiescent centre seems to be correlated with a low RNA content as measured by the size of the nucleoli and the affinity of the cytoplasm for basic dyes such as toluidine blue and pyronin.

Many biologists think that RNA content is related to protein synthesis. Protein synthesis in a meristem is possibly also correlated with rates of DNA synthesis since there are no big differences in cell size within the meristem, and DNA synthesis can therefore be related to cell growth.

This paper describes an investigation of protein synthesis in root meristems by determining the relative rates of incorporation of radioactively labelled sulphate and L-leucine. Sulphate can be used in the synthesis of proteins which contain cystine, cysteine, and methionine. L-Leucine occurs in the hydrolysis products of many plants and is readily incorporated into protoplasmic proteins. The roots used were the primary seedling roots of *Vicia*

faba, *Zea mais*, and *Sinapis alba* and the adventitious roots of *Allium ascalonicum* and of the fern, *Azolla filiculoides*.

SULPHATE INCORPORATION

Plants were arranged so that their roots were in contact with a carrier-free S^{35} sulphate solution 1·0 mc./l. in N/100 sodium chloride containing less than 0·01 mg. sulphur per litre, for from 1 to 4 days. For *Vicia*, *Zea*, and *Allium* air was bubbled through the solution. *Sinapis* seedlings were grown on filter paper dipping in the solution, and *Azolla* plants were floated on the solution. The root tips were fixed in acetic alcohol, sectioned, and covered by a stripping film autoradiographic emulsion as in the experiments on adenine incorporation (Clowes, 1956*b*) except that the sections were not hydrolysed. S^{35} has low energy β particles and makes good autoradiographs like C^{14}. In two of the experiments twelve root apices each of *Zea* and *Vicia* were ground up and extracted in 70 per cent. ethanol. The extract was evaporated at room temperature, redissolved in 0·1 ml. water, and analysed by two-way paper chromatography using as solvents the ethanol/ammonia/water and mesityl oxide/formic acid/water mixtures of Elliot (1954). The residue from the ethanol extract was hydrolysed in 6N HCl at 100° C. for 16 hours, evaporated at room temperature, redissolved in 0·1 ml. water, and analysed similarly. The fate of the S^{35} atoms was determined by making autoradiographs of the chromatograms on X-ray film.

The chromatograms of the hydrolysates and their autoradiographs are illustrated in Fig. 1. Nearly all the radioactivity was concentrated in the cysteine-cystine spot which did not contain enough of the substances to turn purple on spraying with ninhydrin. Weak radioactivity occurred in cysteic acid and in some other unidentified products of cystine. There was also weak radioactivity in methionine and methionine sulphoxide or sulphone. In *Vicia* there were two other weakly radioactive spots from unknown substances. Chromatograms from the ethanol extracts showed strong radioactivity in cysteine-cystine and weak radioactivity in their products. Methionine and its sulphoxide and sulphone did not appear.

In the autoradiographs of sections the density of the silver grains varies from experiment to experiment, but in the apices of all five species it is higher over the cytoplasm than over the background and higher over the nuclei than over the cytoplasm. Over the meristematic cells the autoradiograph is stronger than over the cells which are known to be non-meristematic. Nevertheless, even the non-meristematic cells of the root cap show some incorporation of sulphate, especially into the nuclei. Nuclei of meristematic cells have over them from four to six times as many silver grains as occur over the non-meristematic nuclei of the cap.

In *Vicia*, *Zea*, *Allium*, and *Sinapis* the cells of the quiescent centre have autoradiographs of the same density as the non-meristematic cells of the cap. These quiescent cells stand out conspicuously from the surrounding cells of the meristem. As in the adenine-fed roots, the distal boundary of the quiescent

centre is clearly defined, but at the proximal edge there is a gradual increase in density of silver grains. In *Azolla* the roots have no quiescent centre and the apical cell incorporates sulphate similarly to the surrounding cells of the meristem.

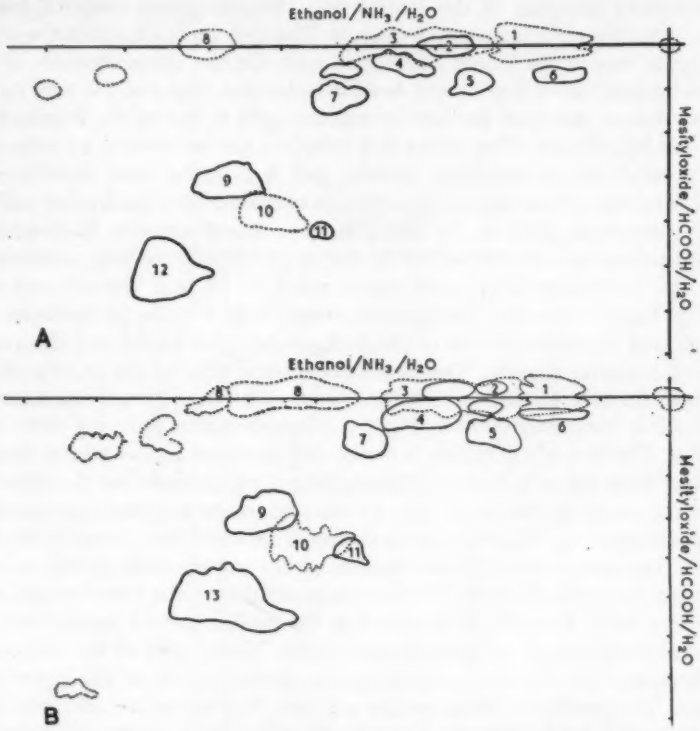

FIG. 1. Chromatograms of hydrolysates of root apices fed with labelled sulphate. (A) *Zea mais*, (B) *Vicia faba*. Ninhydrin spots are shown by whole lines, radioactive spots by dotted lines. The identified spots are (1) cysteine-cystine, (2) cysteic acid, (3) methionine sulphone and/or sulphoxide, (4) glycine, (5) glutamic acid, (6) aspartic acid, (7) alanine, (8) unknown product of cystine, (9) valine, (10) methionine, (11) tyrosine, (12) leucine-isoleucine, (13) leucine-isoleucine and phenylalanine.

L-LEUCINE INCORPORATION

In the first experiments with leucine, seedlings of *Zea* were germinated and when the primary roots were 5 cm. long they were transferred to a solution of L-leucine generally labelled with C^{14} of strength 3·32 mg./l. and of activity 0·2 mc./l. The solution was aerated. After one or two days the apices were fixed in acetic alcohol and autoradiographs were prepared from 6μ sections as before. Four apices were washed and ground in five changes of 70 per cent. ethanol to obtain all the free amino-acids. The residue was hydrolysed in

6N HCl for 16 hours at 100° C. Both the ethanol extract and the hydrolysate were evaporated to dryness at room temperature under reduced pressure and chromatograms run in ethanol-ammonia and mesityl oxide-formic acid. Autoradiographs of the chromatograms were made on X-ray film.

Ninhydrin spraying of the hydrolysate chromatograms revealed twelve spots including eight known amino-acids. The only radioactivity in the chromatogram was in the leucine-isoleucine spot. On the chromatogram of the ethanol extract there was a weak leucine-isoleucine spot and the only radioactivity was in this spot. Its activity was one-fifth of that of the leucine spot from the hydrolysate. This shows that L-leucine can be taken in by roots and incorporated into protoplasmic protein, and that, under these experimental conditions, there is no significant exchange of carbon between leucine and the other compounds likely to be found in microtomed sections. Radioactivity in the sections can be assumed to be due to proteins containing L-leucine.

In the autoradiographs silver grains occurred all over the sections at a density higher than the background except that over large vacuoles the density was the same as that of the background. The nuclei and cytoplasm showed a similar density. The non-meristematic cells of the middle of the cap have the same number of silver grains per unit area as the quiescent centre. The cells immediately surrounding the quiescent centre have 2–5 times that number. The line of cap initials is clearly differentiated by the greater density of silver from the cells of the quiescent centre on one side and the differentiating cap cells on the other side. In the rest of the meristem, proximal to the quiescent centre, there are two cylinders of cells with very dense autoradiograph. The outermost of these consists of the meristematic epidermis and neighbouring cortical layers. The innermost consists of the inner cortical cells and outer stele. Between these cylinders the middle cortical layers have less dense autoradiograph, as does the inner stele. The density of the autoradiograph varies with distance from the apex in the way shown in Fig. 2 for three tissues. The number of silver grains per unit area varies considerably from root to root, and the distance from the apex at which particular stages are reached varies with the width of the root as might be expected (Clowes, 1950), but in all the roots examined the patterns of Fig. 2 are followed. In this work the dimensions are taken from sections after fixing and embedding in paraffin, when some shrinkage occurs. The volumes of cells are calculated on the assumption that the cells are right elliptical cylinders. The changes in cell volume are given in Fig. 3 and in leucine incorporation per cell in Fig. 4. The changes in cell volume recorded by Jensen (1955) in *Vicia faba* roots are much smaller than those found here in *Zea*. His pith cells increased in volume threefold between 1,000μ and 3,000μ from the apex. The discrepancy is at least partially accounted for by the difference in size of the two roots. The change in average cell volume for all tissues in *Pisum* recorded by Brown and Broadbent (1950) is of the same order as that found here.

In a second series of experiments, seedlings of *Vicia faba*, which has a different kind of meristem, were treated similarly. Strong radioactivity in the

hydrolysed material is found in the leucine-isoleucine spot, but there are also two other unidentified spots on the chromatograms with weak radioactivity. Neither of them is demonstrated by ninhydrin spraying. The ethanolic

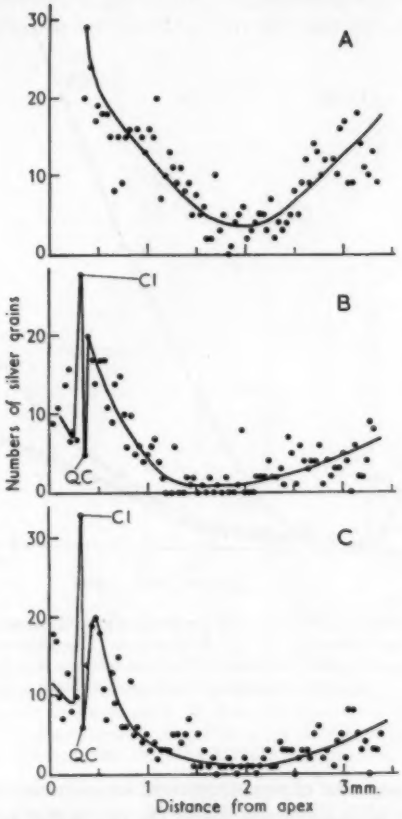

FIG. 2. Relative rates of leucine incorporation per unit volume of tissue in the first 3 mm. of a root apex of *Zea mais*. (A) epidermis, (B) inner cortex and cap, (C) central stele and cap. All results are from the same median section cut 6μ thick from a root exposed to labelled leucine for 48 hours. Ordinates, no. of silver grains in a quadrat $8 \times 4\mu$; abscissae, distance from tip of root; cap initials (CI); quiescent centre (QC). Mean background count 1·3.

extracts show weak radioactivity in the leucine-isoleucine spot. Autoradiographs of the sections show a distribution of the radioactivity similar to that in *Zea* roots. There is a clear quiescent centre surrounded by tissues with more radioactivity, including a plate of cap initials on the distal side. The greatest concentrations of silver grains in the apex are over the meristematic epidermis

and adjacent outer cortex and over the outer stele and inner cortex. As in *Zea* the inner stele and middle cortex have a lower rate of incorporation than the neighbouring tissues at the same transverse level.

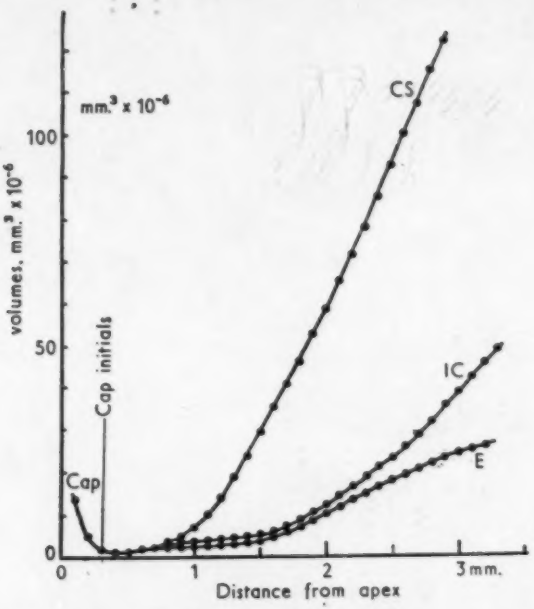

FIG. 3. Change in cell volume (ordinates) in the central stele (CS), inner cortex (IC), epidermis and cap (E), with distance from the root tip, in *Zea mais*. The volumes are calculated from measurements of the mean height of cells in longitudinal sections and the mean long and short diameters of cells in transverse sections.

DISCUSSION

In these experiments the chromatographic analysis shows that most of the S^{35} and C^{14} occurs in compounds insoluble in 70 per cent. ethanol. After hydrolysis of the insoluble residue of sulphate-fed roots most of the S^{35} is found in cysteine-cystine and their products and probably all the rest occurs in methionine and its products. Similarly in leucine-fed roots most of the C^{14} in the hydrolysate of the insoluble fraction occurs in leucine. The apices from which the autoradiographs of sections are obtained are treated with ethanol, benzene, and molten paraffin and, after sectioning, with xylene, ethanol, and water. The radioactivity in the sections is therefore probably due entirely to proteins containing cysteine, cystine, and methionine in plants fed with sulphate or to proteins containing leucine in plants fed with leucine. The density of the autoradiographs is therefore a measure of the incorporation of sulphate or leucine into protein during the period of feeding. The rate of incorporation

is not necessarily the same as the rate of net synthesis since there is evidence (e.g. Jensen, 1956; Gale, 1957) that amino-acids supplied to cells can be exchanged for the same amino-acid already present in the protein without net synthesis occurring. The same reasoning can probably be applied to sulphate incorporation. However, in the present experiments it is assumed that all the essential amino-acids are available from the seed or other organs in con-

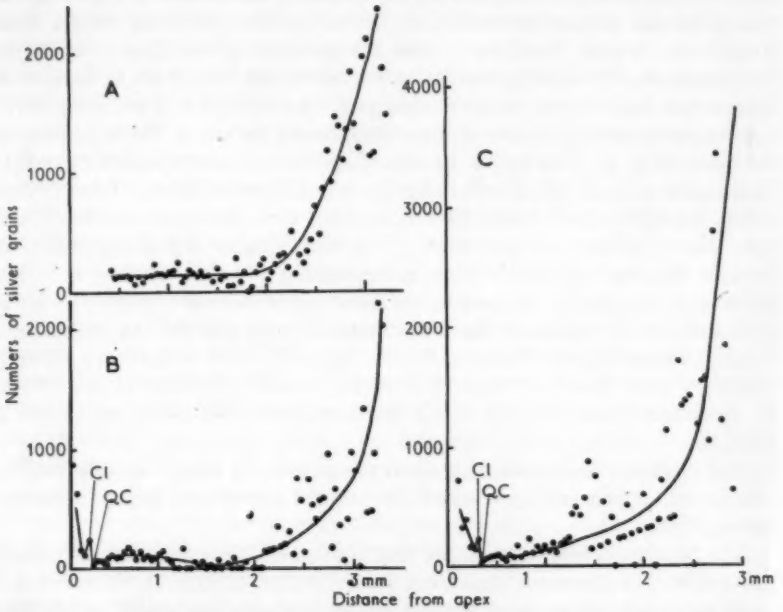

FIG. 4. Diagrams to show relative rates of leucine incorporation per cell in the first 3 mm. of a root apex of *Zea mais*. (A) epidermis, (B) inner cortex and cap, (C) central stele and cap. Ordinates, no. of silver grains recorded in Fig. 2 × cell volume/volume of section under quadrat; abscissae, distance from tip of root; cap initials (CI); quiescent centre (QC).

centrations which are large compared with the externally supplied 0·01 mg./l. of sulphur or 3·3 mg./l. of leucine. Under such conditions net synthesis can occur and part of the radioactivity measured is probably due to net synthesis, though part may be due to other kinds of incorporation.

Non-meristematic cells incorporate sulphate into the protein of the nucleus, but at about a fifth of the rate of meristematic cells. This difference is similar to the difference found in DNA synthesis in nuclei of meristematic and non-meristematic cells (Clowes, 1956*a*), but the data are not strictly comparable, since, in the experiments on DNA synthesis, the roots were hydrolysed to remove RNA after feeding with adenine, and this will almost certainly also remove some of the DNA and leave some RNA. The cells of the quiescent centre are similar to non-meristematic cells in the uptake of sulphate.

Pelc and Howard (1952) reported that inorganic S^{35} was incorporated into the nuclei of only some of the cells of *Vicia* roots. The difference between their observations and the present ones is probably due to the difference in treatment. Pelc and Howard's roots were hydrolysed, those used in this work were not.

Non-meristematic cells also incorporate L-leucine into protoplasmic protein. The meristematic cells around the quiescent centre have a much higher rate of leucine incorporation than do the cells of the quiescent centre. Both sets of experiments therefore confirm the existence of a quiescent centre to the meristem. Proximal to the quiescent centre the rate of incorporation of leucine per unit volume of tissue rises and then falls off and becomes lower than in the quiescent centre at 800–1,000μ from the tip in *Zea* according to the tissue (Fig. 2). This fall in the rate of synthesis is accompanied by reduction in the rate of cell division and the onset of vacuolation, which occurs before the cells stop dividing. The relation between rate of incorporation and cell division and vacuolation in this region also explains why at any particular level in the meristem the highest incorporation per unit volume is in the epidermis and outer cortex and in the inner cortex and outer stele. The inner stele and middle cortex are the first tissues to begin and end vacuolation and the first tissues to stop division (Clowes, 1950). The rate of synthesis per unit volume of protoplasm is presumably smaller in cells which are in the process of vacuolation than in cells which remain unvacuolate if the expansion is similar.

The relatively high number of silver grains over the distal cap cells may be due partly to their moribund condition and the presence of micro-organisms among them.

The number of silver grains per unit volume of tissue falls to nearly background level at 1,600μ–2,000μ from the tip in *Zea*. It then rises between this level and 3,500μ from the tip, where present experiments end (Fig. 2). This increase occurs in the region where most of the cell growth occurs and where mitosis has practically stopped. The rate of incorporation per unit volume does not reach the rate found in the meristem immediately round the quiescent centre, but the rate of incorporation per cell at 3,000μ is 15 times greater in the epidermis, 25 times greater in the inner cortex, and 50 times greater in the central stele (Fig. 4).

Other work on protein synthesis in root meristems has been done by measuring the amount of protein present in transverse slices of apices 200, 400, or 506μ thick (Brown and Broadbent, 1950; Erickson and Goddard, 1951; Jensen, 1955). This determines the change in protein content with age and differentiation. The use of slices also differs from the present experiments because the availability of leucine is different. Interpretation of the present experiments assumes that labelled leucine is available to all parts of the apex in amounts which do not alter significantly the amino-acid balance. It also assumes that fixation is equally good for all kinds of protein. Autoradiography has an advantage to the anatomist over the use of thick slices in showing the

site of protein synthesis more precisely. The use of transverse slices is not open to the anatomist because there are variations in the metabolic state and the volume of the cells with distance from the axis as well as along the axis. These are shown in the graphs (Figs. 2, 3, and 4). Variations in the state of differentiation at any one level also make it difficult to put the data on a protein weight basis. It is impossible to detect the changes of metabolism around the quiescent centre by using thick slices, and these changes are important in studying the structural development of roots. Autoradiography has the disadvantage that it cannot detect a negative rate of synthesis. Also a cell situated at 1 mm. from the quiescent centre at the beginning of the experiment will be at some other position relative to the quiescent centre at the end of the experiment. The number of silver grains will therefore depend on rates of incorporation at a range of positions.

Jensen (1955) differs from Brown and Broadbent (1950) in his conclusion on the pattern of protein increase along the length of the apex. Jensen holds that protein nitrogen per cell increases between 500μ and 1,000μ from the tip and falls between 1,000 and 3,000μ in *Vicia*. Brown and Broadbent hold that protein nitrogen per cell increases between 300μ and 5,000μ in *Pisum*. As *Pisum* roots are smaller than *Vicia* primary roots it might be expected that Jensen's fall in protein, if it is real, would be detected in the range 300μ–5,000μ. Jensen believes that the difference between his results and the others is due to the different ways of measuring cell volume. This seems unlikely. A more probable explanation is that different conditions of growth existed.

Erickson and Goddard's (1951) curve of protein nitrogen per cell in *Zea* is similar in shape to that of Brown and Broadbent. The increase in protein nitrogen per cell recorded by Brown and Broadbent is 11-fold; that recorded by Erickson and Goddard is 2-fold; and that recorded by Jensen is 4-fold. The rate of incorporation of leucine per cell in the present experiments increases 15-fold in the epidermis, 25-fold in the inner cortex, and 50-fold in the central stele between the meristematic cells around the quiescent centre and the elongating cells 3 mm. from the tip. This wide variation in the rate of incorporation may be due to a change in the leucine content of the protein with age of the cells. Such changes in protein composition have been noted by Morgan and Reith (1954). Changes in protein preservation by fixation and subsequent treatment, or in exchangeability of leucine involving no net synthesis, are also possible. But even if there are no such changes and the figures are a good measure of relative net synthesis the results are not inconsistent with the other work discussed above. The use of thick slices might easily halve the real change in protein nitrogen per cell. If the protein of the meristematic cells near the quiescent centre doubles its mass in 2 days, the protein of cells 3 mm. from the tip will double its mass in one day if the cells at 3 mm. have 20 times the protein mass and 40 times the rate of synthesis of cells near the quiescent centre. Such a small change in the rate of protein synthesis per unit mass of protein is not beyond the bounds of possibility. More work on these lines may well prove useful.

ACKNOWLEDGEMENTS

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Fat Metabolism in Germinating *Citrullus vulgaris*. Part II[1]

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SUMMARY

Breakdown of fat in watermelon seedlings germinated in the dark occurs in essentially the same way as in the light. Free fatty acids do not accumulate and the composition of the lipid remains almost unchanged during its rapid utilization.

The low weight of 'protoplasmic lipid' remaining in the etiolated cotyledons after the storage fat has disappeared contains a lower proportion of linolenic acid than that from green cotyledons of a corresponding age. The weight of phosphatide (lipid P) per cotyledon increases both in light- and dark-grown seedlings until 8 days, thereafter declining in the dark but continuing to rise in the light. Phosphatide and glyceride fractions from the same fat resemble each other fairly closely in fatty acid composition.

INTRODUCTION

FATTY acid breakdown in watermelon seedlings grown in the light has already been described (Crombie and Comber, 1956) and the first part of the present communication compares the metabolism of seedlings grown in the dark. Preliminary data on phosphatide (lipid P) synthesis and on the fatty acid composition of crude glyceride and phosphatide fractions from etiolated and green cotyledons are included.

MATERIALS AND METHODS

Plants were grown under conditions similar to those described previously (Crombie and Comber, 1956), light being excluded by covering the seed boxes with a light-tight box. The main experiment was carried out in January 1956 and plants were sampled at intervals from 0 to 14 days (later stages could not be examined owing to bacterial attack on the starved plants). Seedlings were removed before sunrise to a dark room, separated into cotyledons and 'rest of plant', and killed by immersion in boiling solvent. These operations were carried out in a dim green light (40-watt bulb covered by an Ilford GB 908 filter).

For the main experiment the methods used for the determination of fresh weight, dry weight, starch, sucrose, reducing sugars, and lipid fractions were identical with those described earlier (Crombie and Comber, 1956). Phosphatide analyses were carried out on material which had been killed by immersion in boiling ethanol (10 mins.). This solvent is preferred for this purpose to the acetone used in the normal analysis because of its superior ability to split the

[1] The paper published under the same title in *J. Exp. Bot.* 7, 166 (1956), is regarded as Part I.

loose complexes formed between phosphatides and other cell components (Meara, 1955): phosphatides can then be extracted by normal lipid solvents. The killed material was cooled, homogenized, and set aside overnight. The next day the suspension was filtered and the precipitate washed alternately with ethanol and diethylether (final mixture 1:2 v/v). The solvents were evaporated at low temperature in a stream of nitrogen and the remaining aqueous emulsion extracted three times with light petroleum (b.p. 40–60°). Evaporation gave the weight of lipid extract. Values obtained by this method and the original dry Soxhlet extraction method for the weight of lipid in dry *Citrullus* seeds agreed well: on actively growing tissue the wet method extracted somewhat more material than the dry method.

Crude separations of the lipid extract into glyceride (acetone soluble) and phosphatide (acetone insoluble) fractions were carried out by method Ja 4–46 (American Oil Chemists' Society, 1953). Phosphorus was determined on lipid fractions by a technique for which we are indebted to Mr. H. H. Hutt: 0·5 g. or less of lipid was saponified in a platinum crucible with 0·5 ml. 30 per cent. aqueous NaOH and 1 ml. ethanol and then ignited. The resulting ash was dissolved in water and phosphorus determined colorimetrically on a sample (Snell and Snell, 1949). The weight of phosphatide was calculated as 23·4 × wt. of P (this assumes that the phosphatide consists only of a cephalin containing two linoleic acid radicals: other types are undoubtedly present so the figure obtained is only approximate).

RESULTS

Seedlings germinated in the dark

Morphology and cytology. In the dark the cotyledons never expand or turn green as they do in the light, but between the 4th and 7th days a yellow pigment is produced. The root system is reduced, but hypocotyl development is rapid, so that at comparable ages plants grown in the dark are at least twice the height of those grown in the light.

In the soaked seed storage fat in the form of large globules fills the cotyledon cells. As germination progresses these large globules are gradually reduced in size until by the 14-day stage they have disappeared.

General components. Fresh and dry weight changes in the cotyledons and 'rest of the plant' are shown in Table I. Total dry weight declines steadily between 4 and 14 days and during this time there is appreciable redistribution of material from the cotyledons to the 'rest of the plant'. Subsidiary experiments showed that the slight increase in dry weight noted previously at an early stage in germination (Crombie and Comber, 1956) is not statistically significant. Analyses of lipid and carbohydrate components (Table I) indicated that depletion of the cotyledonary storage oil is not accompanied by any appreciable increase in total starch, sucrose, or reducing sugars. A similar result was found in seedlings germinated in the light. Most of the increased dry weight of the 'rest of the plant' is due to 'residual dry weight' (dry weight minus determined constituents) and, since this fraction consists largely of

cell-wall materials and protein, this increase can be correlated with the rapid production of new cells until the 11th day.

TABLE I

Germination of seedlings in the dark. Components per 100 plants

(*Each figure is the mean of duplicate samples which agreed well*)

Age (days)	F. wt. (g.)	Dry wt. (g.)	Lipid extract % of dry wt.	Combined fatty acid (g.)	Redcg. sugars (mg.)	Sucrose (mg.)	Starch (mg.)	Resid.* dry wt. (g.)
Cotyledons								
0	4·43	4·23	49·2	1·867	2·9	88·7	5·4	2·06
4	6·71	3·70	51·6	1·573	2·0	66·1	8·5	1·72
7	9·82	1·98	31·1	0·463	14·5	56·9	14·8	1·27
11	11·33	1·13	9·9	0·082	8·0	18·6	15·0	0·98
14	13·78	1·06	5·0	0·041	6·8	4·9	22·8	0·97
'*Rest of plant*'								
4	3·93	0·34	17·5	—	8·9	5·4	4·5	0·26
7	64·13	1·89	1·3	—	45·2	6·7	30·6	1·79
11	96·31	2·28	0·8	—	7·1	2·5	23·3	2·23
14	101·68	2·18	0·9	—	3·3	0·9	18·2	2·13

* Dry wt. minus lipid and determined carbohydrates.

Water-soluble acidity was determined in 0- and 5-day old cotyledons (14·1 and 43·3 mg. equivs./100 seedlings respectively), but despite this large increase in titratable acidity, the pH of the tissue changed only from 5·8 to 5·5.

Components of the lipid extract. Only the lipid extract from the cotyledons was examined in detail since the weight of this fraction in the 'rest of the plant' was too low for analysis. Non-saponifiable material remained at a low level throughout the period studied, but formed a much greater proportion of the lipid from the late germination stages (about 20 per cent. at 14 days compared with 0·2 in the seed). This fraction contains the yellow pigment which develops as germination proceeds.

The amounts of non-volatile free fatty acids are very low at all stages, whether determined by titration of the crude lipid extract or by the weight of sodium carbonate extractable material (maximum value at 7 days was 3·8 mg./100 plants). It was noticed that the former method tends to give higher results than the latter; a similar difference was noticed with light-grown material (Crombie and Comber, 1956) and possible causes of the discrepancy were discussed.

Volatile acids were determined by steam distillation of the dry seeds and 5-day-old seedlings: the results obtained were 1·2 and 2·7 mg. equivs./100 seedlings respectively, indicating that volatile acids do not accumulate during rapid lipid breakdown. This result is similar to that recorded for seedlings germinated in the light.

Combined fatty acids form a major part (> 90 per cent.) of the lipid extract from dry seeds, indicating the predominance of triglycerides, but by the

14-day stage they constitute only about 40 per cent. The fatty acids present in the seed oil are palmitic (C_{16}), stearic (C_{18}), oleic, and linoleic acids, but by the 7-day stage it is possible to detect a small quantity of an additional unsaturated acid (Fig. 1). This was identified from its chromatographic behaviour (speed of elution and destruction by alkaline permanganate) and UV spectrum (λ_{max} 268, subsidiary maxima at 258 and 278 mμ after isomerization) as a

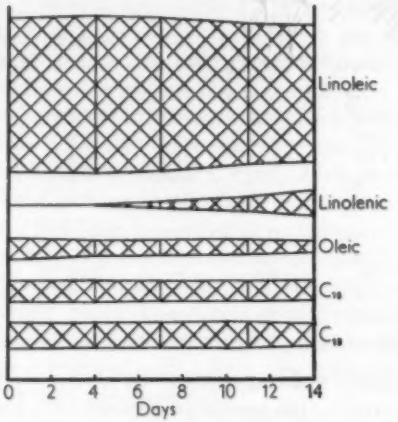

FIG. 1. Changes in percentage composition (g./100 g. total fatty acids) of the fatty acids during germination in the dark.

non-conjugated octadecatrienoic acid.[1] The weight of this acid reaches a maximum by the 7th day and then declines. Between 7 and 14 days there is a small but definite change in percentage composition of the oil, although there is no evidence of fatty acid interconversions. This is due to a change in relative amounts of the unsaturated acids—oleic and linoleic acids become less important constituents whereas linolenic acid forms finally over 12 per cent. of the total fatty acids. During this time all acids are declining in weight per cotyledon and the changes in composition result from differences in the rates of loss, linolenic acid being removed at a slower rate. This change in composition of the oil is considerably less marked than that which occurs during germination in the light (Crombie and Comber, 1956) where in the fat from the final stages (16 days) linolenic acid forms about 63 per cent. of the total fatty acids.

It was considered possible that such a change in fat composition during depletion might result from a difference in the rates of breakdown of glyceride and phosphatide fatty acids. If glycerides were removed more rapidly the composition of the remaining fatty acid mixture would approximate more closely to that characteristic for the phosphatides. It is well known (e.g.

[1] This acid will be referred to as linolenic although the position of the double bonds in the chain is unknown.

Hilditch, 1956) that glycerides and phosphatides derived from the same tissue differ somewhat in fatty acid composition. In order to obtain more information a preliminary investigation into the changes in phosphatides during germination was undertaken. This study included examination of material grown both in the light and in the dark.

TOTAL PHOSPHATIDE CHANGES DURING GERMINATION

The results of lipid phosphorus analyses are shown in Fig. 2. A rather surprisingly low weight of phosphatide was detected in the seed oil, but by the 8th day this had increased considerably both in the light- and in the dark-

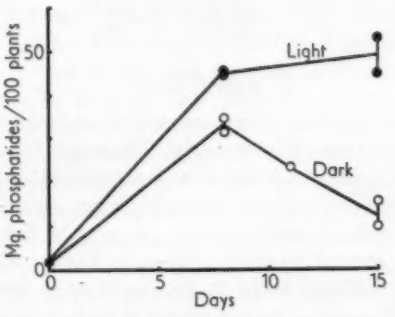

FIG. 2. Changes in total phosphatide per 100 plants (cotyledons) during germination.

●——● germinated in the light.
o——o germinated in the dark.

grown cotyledons. Although after the 8th day there was a decline in phosphatide in the dark, the proportion of phosphatide in the lipid continued to rise as germination proceeded, achieving a value of about 25 per cent. in the 14-day stage compared with a value of about 0·1 per cent. in the ungerminated seed.

Fatty acid analyses of crude phosphatide and glyceride fractions

Lipid extracts were obtained from two samples of cotyledons, one grown for 11 days in the dark and the other for 14 days in the light. A crude separation by acetone solubility into glyceride and phosphatide fractions gave the results shown in Table II. Phosphorus determinations on the fractions indicated that better separation was achieved in the case of the light-grown material. Each fraction was then saponified in the usual way and the fatty acids weighed and analysed. Differences in fatty acid composition are not great and this agrees with published results for other materials (Hilditch, 1956). It is interesting that the phosphatides from light- and dark-grown cotyledons are more similar than the glycerides.

TABLE II

Phosphatide and glyceride fractions from light- and dark-grown cotyledons

Components per 100 plants

	11-day dark		14-day light	
	Glyceride	Phosphatide	Glyceride	Phosphatide
Total wt. (mg.)	63·5	29·2	109·2	60·6
Fatty acid (mg.)	25·0	11·7	38·5	27·3
Phosphorus (mg.)	0·32	0·84	0·25	1·46
Fatty acid/P (mol./atom)	8·7	1·6	17·2	2·1
Palmitic (g./100 g. fatty acid)	8·1	13·8	7·5	19·5
Stearic ,, ,,	10·6	12·2	6·5	9·5
Oleic ,, ,,	8·3	0·0	3·5	4·0
Linoleic ,, ,,	52·2	44·8	19·8	23·1
Linolenic ,, ,,	20·8	29·2	62·7	43·9

DISCUSSION

Seedlings grown in continuous darkness show interesting morphological differences from those grown in the light. The cotyledons never expand, root development is reduced, and there is great elongation of the hypocotyl. Despite this very different pattern of development, stored fat disappears from the cotyledons at approximately the same rate as in light-grown plants and there is again no accumulation of sugars or starch. The use of etiolated material enables a balance sheet to be constructed and this is shown in Table III. There is a steady loss of material from the cotyledons and, until the 11-day stage, about half of this is transferred to the 'rest of the plant', where it appears mainly as residual dry weight (largely protein and cell-wall constituents). The remainder is lost in respiration. After 11 days carbon is lost from the 'rest of the plant' at a faster rate than material is being translocated into it from the cotyledons. By this stage plants grown in the light have expanded green cotyledons, which are capable of photosynthesis, and the total dry weight of the plant is increasing.

TABLE III

Distribution of carbon between the different parts of seedlings grown in the dark

Mg. C/100 plants

	Cotyledons				'Rest of plant'				
Stage	Carbohyd. (starch, sucrose, and reducing sugars)	Lipid	Resid. dry wt. (calc. as carbohyd.)	Total	Carbohyd.	Lipid	Resid. dry wt.	Total	Whole plant Total
0–4 days	−9·1	−133·0	−148·0	−290·1	+8·4	+46·3	+117·0	+171·7 (59·0)*	−118·4 (41·0)†
4–7 days	−4·3	−1007·0	−199·0	−1210·3	+28·5	−27·7	+679·0	+679·8 (56·5)	−530·5 (43·5)
7–11 days	−19·8	−394·0	−130·2	−544·0	−21·9	+4·5	+195·0	+168·6 (31·0)	−375·4 (69·0)
11–14 days	−3·2	−44·2	−3·9	−51·3	−4·6	−0·2	−42·8	−47·6	−98·9

* Percentage of reserves in cotyledons transferred to the 'rest of the plant' during this period.
† Percentage of reserves in cotyledons lost in respiration during this period.

During rapid fat loss there is, as in light-grown seedlings, remarkably little change in lipid composition (the somewhat faster rate of loss of oleic acid recorded previously was not confirmed in this investigation) and it is interesting that cytological examination showed that the large globules of fat present in the ungerminated seed are gradually reduced in size, suggesting that they may be homogeneous. An active lipase has been demonstrated both in the dry seed and in later seedling stages, but there is no evidence that this enzyme functions in intact cotyledons, since free fatty acids did not accumulate.

After disappearance of storage fat etiolated *Citrullus* cotyledons have a lower concentration of linolenic acid in their 'protoplasmic fat' than green cotyledons (Crombie and Comber, 1956). A similar effect was found in germinating *Helianthus annuus* cotyledons (Hardman, unpublished) and in the foliage leaves of *Vicia faba* seedlings (Crombie, 1956). Since light stimulates production of phosphatides, both in *Citrullus* and in other seedlings (Smith, 1947), this difference could be explained by preferential synthesis in the light of phosphatides richer in linolenic acid than the corresponding glycerides. Analysis shows, however, that there are only minor differences in fatty acid composition between phosphatides and glycerides and that the proportion of phosphatide in the protoplasmic fat is similar in light- and dark-grown cotyledons. The difference can more convincingly be attributed to the promoting effect of light on the synthesis of chloroplasts, whose fats are known to contain a high proportion of linolenic acid (Crombie, 1958).

Since it has been shown (Williams and Sharma, 1954) that cotyledon growth in *Citrullus* is due almost entirely to expansion of existing cells, it is tempting to associate the earlier increase in lipid phosphorus, which occurs both in the light and in the dark, with synthesis of mitochondria. These organelles are known to multiply in germinating seeds (Geneves, 1956, Akazawa and Beevers, 1957 *a* and *b*) and are able to synthesize phosphatides (Mazelis and Stumpf, 1955). The correlation between respiration rate (Williams and Sharma, 1954) and phosphatide synthesis in *Citrullus* during the early stages of germination is in agreement with this idea. If this supposition is correct some linolenic acid is probably present in the mitochondria, since the weight of this acid per etiolated cotyledon closely parallels that of the lipid phosphorus.

ACKNOWLEDGEMENTS

We thank the Ferry Morse Seed Company of California for their generous gifts of *Citrullus vulgaris* seeds. One of us (E. E. H.) acknowledges the receipt of a maintenance grant from the University of Southampton. We are grateful to Professor W. T. Williams for his interest and encouragement in this work.

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Fat Metabolism in the West African Oil Palm (*Elaeis guineensis*)

PART III. FATTY ACID FORMATION IN THE MATURING EXOCARP

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SUMMARY

Accumulation of fat in the oil-palm exocarp is delayed until the kernel has almost finished developing (at about 19 weeks after pollination) and is then extremely rapid, a major part of the lipid being formed within a single week. Throughout the period studied (8 to 20 weeks after pollination) the fat-free dry weight remains approximately constant and carbohydrates (starch, sucrose, and reducing sugars) do not accumulate either prior to or during fat formation.

Immature exocarps contain only a low proportion of fat (about 1 per cent. of the dry weight) and this 'protoplasmic' lipid has a different fatty acid composition (major components palmitic and linoleic acids accompanied by smaller amounts of stearic and linolenic acids) from the later-formed oil (major components palmitic and oleic acids with smaller amounts of stearic and linoleic acids). There is no evidence of fatty acid interconversions at any stage of development.

INTRODUCTION

THE synthesis of fatty acids in the oil-palm kernel, which produces palm-kernel oil, has already been studied (Crombie, 1956) and the present communication describes the formation of fatty acids in the fruit coat, which produces palm oil. Palm oil is relatively unsaturated and is composed almost entirely of acids of chain length C_{16} and C_{18}. It therefore forms an interesting contrast with the unusual kernel oil, which is highly saturated and contains a high proportion of shorter-chain acids (major component C_{12}).

MATERIALS AND METHODS

Exocarp material was obtained from the same fruits as those used to study fat synthesis in the kernel. Sampling was carried out in Nigeria by members of the staff of the West African Institute for Oil Palm Research and material was preserved in acetone for transport to England (for full details see Crombie, 1956). Exocarps from only two of the original three trees selected for study were examined (trees 6–140 and 6–173), these being chosen as examples of slow-maturing and somewhat faster-maturing types. Stages in maturation from 8 to 20 weeks after pollination were analysed for dry weight, weight of

lipid extract, starch, sucrose, reducing sugars, and components of the lipid extract, using techniques already fully described (Crombie, 1956); each sample contained the total exocarp material from 20 fruits.

RESULTS

Appearance. Examination of fresh material (sent to England by air) showed that in the early stages of development fruits are already nearly full-size, pale green or yellow in colour at the base, and not notably oily. In contrast mature exocarps are slightly larger, deep orange in colour, and very oily (full descriptions are given by Blommendaal (1925), Bunting *et al.* (1927), and Tobler and Tobler (1921)). The appearance had naturally altered with preservation, but it was observed that tissue from tree 6–173 retained its immature characteristics until 16 weeks after pollination, whereas the 20-week stage was deep orange and very oily. The 19-week stage was intermediate in appearance. Fruits from tree 6–140 still showed the immature characteristics at 19 weeks after pollination and it was suspected that exocarps from this tree were not fully mature even in the last stage examined (20 weeks after pollination).

Blommendaal (1925) observed that fat appears first as small droplets in the cells surrounding the vessels; later these droplets increase in size until the cells are filled. Oil accumulates first at the base of the fruit and finally at the periphery and during this time chlorophyll, which is obvious in the young fruits, disappears and is replaced by carotene.

General components. In both series dry weight remains fairly constant between 8 and 19 weeks after pollination (Table I), although there is a slight overall increase in exocarp dry weight in tree 6–140 which is almost masked by fluctuations. Fruits were selected from the centre of bunches, since position markedly affects the amount of oil produced (Blommendaal, 1925), but, despite this, variation between samples is obviously large. In both trees there is a marked increase in dry weight at the 20-week stage, which must be due to rapid translocation of material into the fruits. Blommendaal (1925) found that fruits detached while immature would not ripen.

The weight of lipid remains low and fairly constant in exocarps from both trees between 8 and 16 weeks after pollination. The weights of fat per fruit are similar in the two series, but fat forms a higher percentage of the dry weight in tree 6–173 (Table I): this difference reflects the greater weight of fat-free tissue in tree 6–140. Such low figures for lipid content indicate that no fat accumulation occurs until a late stage of development, a situation rather different from that in the corresponding kernels, in which oil starts to accumulate by about 13 weeks after pollination. By the 19-week stage the fat content of the exocarp has risen in both trees, though to a greater extent in tree 6–173, where external appearance of the fruit also indicated greater maturity.

Sucrose was not detected at any stage in either series of samples. Reducing sugars and starch show considerable fluctuations in amount. Residual dry weight (dry weight minus lipids and determined carbohydrates) remains approximately constant throughout the period studied and most of the

marked increase in dry weight, which occurs at maturity, is due to oil accumulation.

TABLE I

Changes in general components during maturation of oil-palm exocarp

Dry wt. and residual dry wt. in g./20 fruits.
Other components in mg./20 fruits.

Weeks from pollination	Dry wt.	Lipid extract (% of dry wt.)	Starch	Reducing sugar (as glucose)	Residual dry wt. (dry wt. minus determined constituents)	Non-saponifiable material	Total fatty acids (chromatographic)
Tree 6–140							
8	10·7	0·9	53	1,217	9·36	2·6	38·8
10	10·7	0·7	62	635	9·90	8·0	32·4
11	12·8	0·7	72	341	12·33	2·5	37·6
12	10·9	0·5	85	9	10·79	7·8	15·5
14	12·1	0·8	68	320	11·61	29·5	49·5
15	13·8	0·6	46	424	13·28	7·2	32·3
16	13·9	0·4	105	410	13·28	10·9	—
19	13·2	3·9	72	251	12·39	20·4	315·2
20	30·3	56·0	106	254	13·01	432·0	12,685·0
Tree 6–173							
8	6·5	1·3	27	343	6·00	36·6	—
12	5·2	1·4	14	94	5·16	5·7	30·6
16	6·7	1·3	16	430	6·20	15·9	42·9
19	6·8	22·4	—	—	—	10·5	933·4
20	33·9	70·5*	116	170	9·69*	149·6	18,783·0

* Lipid was incompletely extracted from this sample.

Non-saponifiable material shows considerable variation in amount in the immature stages, but in both trees there is a marked increase at 20 weeks as storage oil accumulates. The extract from immature fruits is light in colour, but that from mature fruits is deep orange and contains the carotenoid developed in the final stages of ripening.

No special precautions were taken during sampling in West Africa to prevent the rapid rise in acidity, which results from damage to oil-palm exocarps (Vanneck *et al.*, 1951), and no attempt was made to analyse free and combined fatty acids separately. Blommendaal (1925) has, however, shown that, when appropriate precautions are taken during sampling, the acid content of palm oil remains low and approximately constant from 30 days before ripening until maturity. A similar low content of free acids characterizes maturing palm kernels (Crombie, 1956).

Total fatty-acid weights (obtained chromatographically) remained at a low level until the 16-week stage and then increased rapidly until 20 weeks after pollination (Table I). In the early stages fatty acids form a relatively low percentage of the lipid extract, but finally they constitute 75 per cent. or more.

Composition of the total fatty acids. Not all the fatty acids characteristic of mature palm fruits (palmitic, stearic, oleic, and linoleic) are present with certainty in the oil from immature fruits; oleic acid, which is a major component

of the ripe oil, occurs only in traces in the immature fruits (Table II). The oil from immature exocarps also contains an unsaturated fatty acid, which was shown to be probably an octadecatrienoic acid, and this was confirmed by recovering the appropriate chromatographic fraction and examining the acid

TABLE II

Composition of the total fatty acids

(g./100 g. total fatty acids)

Weeks from pollination	Saturated acids				Unsaturated acids		
	C_{14}	C_{16}	C_{18}	C_{20}	Oleic	Linoleic	Linolenic
Tree 6–140							
8	Trace	59·6	8·6	Trace	4·2	24·4	3·2
10	,,	48·0	6·6	,,	2·6	30·8	12·1
11	,,	62·8	8·4	,,	0·7	26·4	2·0
14	,,	54·0	6·8	,,	0·0	33·0	6·5
15	,,	57·5	9·0	,,	0·0	23·2	10·5
19	,,	43·9	6·7	,,	35·1	13·8	0·7
20	,,	41·0	9·3	,,	38·5	11·2	0·0
Tree 6–173							
12	Trace	57·5	7·0	Trace	0·9	29·6	5·2
16	2·8	67·0	7·6	1·0	0·0	20·3	1·5
19	0·2	55·7	5·6	0·5	28·2	9·7	0·0
20	0·4	45·5	7·8	0·6	34·0	11·8	0·0

which it contained by UV spectrophotometry after isomerization (λ_{max} 268 mμ; subsidiary maxima at 258 and 278 mμ). Unsaponified oil from two immature stages (14 and 15 weeks—tree 6–173) was also examined by UV spectrophotometry in order to determine whether this octadecatrienoic acid was conjugated or non-conjugated. The results showed the presence of 0·2 and 2·8 per cent. respectively of total triene in the lipid extracts after isomerization, but only traces of conjugated material were present before isomerization. The acid will therefore be referred to as linolenic acid, although there can be no certainty on the position of the double bonds. It proved impossible to detect this acid (either chromatographically or spectrophotometrically) in the oil from mature exocarps but it was found (by both methods) in the 19-week stage of tree 6–140 in which oil had just started to accumulate. Analysis of palm oil by low-temperature crystallization (Hilditch *et al.*, 1947) has revealed the presence in it of 0·4 per cent. of linolenic and 1·8 per cent. of hexadecenoic acids. In addition to the acids already mentioned, small amounts of myristic, arachidic, and longer-chain acids, saturated and unsaturated, were found both in mature and immature fruits.

As the tissue matures oil accumulates rather suddenly and each acid, except linolenic acid, increases in total weight; there is therefore no evidence of fatty acid interconversions. The most interesting feature is the change in composition of the oil at the 19- and 20-week stages. Oleic acid, present only in traces

in the immature oil, forms a major fraction of the new exocarp oil whereas palmitic, linoleic, and linolenic acids are present in lower concentration.

DISCUSSION

One of the most interesting features of fruit maturation in the oil palm is the extremely rapid increase in dry weight of the exocarp during the final weeks of ripening, which contrasts with the slow regular addition of dry weight to the kernel (Fig. 1). The development of oil-palm fruits can be divided into

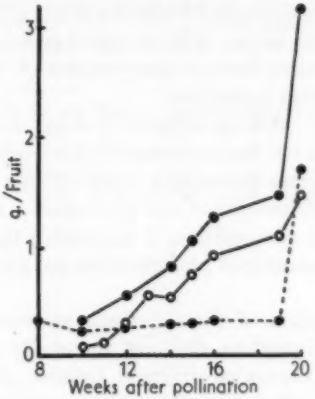

FIG. 1. Comparison of the development of kernel and exocarp in tree 6–173 (dry weights).

●——● Kernel + exocarp
o——o Kernel
● - - ● Exocarp

three main phases which are similar to those described for other stone fruits such as cherry and peach (e.g. Nitsch, 1953). In the first phase, which is completed in the oil palm by about 10 weeks after pollination, endocarp and exocarp are differentiated, whereas the endosperm is as yet undeveloped. In the second phase (from about 10 to 19 weeks after pollination) little further change occurs in the endocarp or exocarp, but cells form in the endosperm which increases steadily in dry weight. In the third phase, which starts when endosperm development is nearly complete, there is a sudden influx of considerable quantities of material into the fruit and most of this appears as oil in the exocarp. Yampolsky (1922) has shown that there are vascular connexions between endosperm and exocarp, and competition for translocated material between these two tissues could explain why final development of the exocarp is delayed until kernel development is almost complete. Tukey (1933) observed that faster pericarp development in cherry was correlated with embryo abortion, suggesting a similar competition.

A second feature is that carbohydrate does not accumulate in the exocarp

prior to the sudden rapid increase in its fat content. There is evidence that exocarp fat is derived entirely from translocated material and this is in agreement with Blommendaal's observation that detached immature fruits would not ripen. Since it seems unlikely that large quantities of fat are translocated, exocarps probably contain a highly active conversion system.

The difference in fat composition of immature and mature exocarp tissue is most readily explained if it is assumed that the early fat is a 'protoplasmic' lipid, synthesized in phase one, remaining unaltered until the large final accumulation of exocarp oil of different composition. The main interest lies in explaining why a single tissue should produce fats of different composition at different developmental stages. Mature and immature lipid is similar in total degree of unsaturation, but the distribution of unsaturation between mono-, di-, and triene acids is different.

It is possible that fatty acids are differently distributed between glyceride and phosphatide molecules in the two stages. 'Protoplasmic' fats are generally rich in phosphatides, whereas storage oils consist almost entirely of glycerides. Since it is known that phosphatides and glycerides derived from the same source differ in fatty acid composition, it is possible that the present results reflect a difference in proportion of phosphatides and glycerides in mature and immature oils.

A more likely explanation of the difference in fatty-acid composition is that the two fats are formed in different metabolic environments, either because their synthesis occurs at different cellular sites, or because their synthesis is affected by an alteration of internal conditions with time. Nothing is known of the intracellular distribution of the original 'protoplasmic' lipid, but it may be significant that immature tissue contains chlorophyll, since linolenic acid, which is present in immature fat, is characteristically found in chloroplasts (Crombie, 1958).

If synthesis is occurring at the same site, changes being due to an alteration in the environment with time, it is obvious that the most striking difference between mature and immature tissue lies in the rate of addition of fat precursor. It has been suggested (Simmons and Quackenbush, 1954) that unsaturated acids are formed in plants in the sequence oleic $\xrightarrow{-2H}$ linoleic $\xrightarrow{-2H}$ linolenic. If each of these radicals can be removed rapidly from the enzyme system a sudden increase in rate of production of oleic radicals might bring about a shortage of the hydrogen acceptor required for the later desaturation stages. This would result in a higher concentration of oleic acid in rapidly formed fat. Further speculation must, however, be delayed until there is more information on the systems involved.

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Fatty Acids in Chloroplasts and Leaves

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SUMMARY

The fatty acid composition of green and white leaf tissue in *Acer negundo*, *Zea mais*, and *Ilex aquifolium*, of green and yellow tissue in *Ligustrum ovatifolium* and of etiolated and green tissue in *Vicia faba* has been determined. The mesophytic green leaves examined show a general similarity in fatty acid composition, characterized by a high concentration of non-conjugated octadecatrienoic acid. Chloroplasts were isolated from *Vicia* and *Acer* and contain an even higher concentration of this acid and only traces of non-conjugated octadecadienoic acid. Conjugated diene and triene acids occur in traces in chloroplasts, but are also found in non-green leaf tissue. The fats of non-green leaves are in general more saturated than those from green tissue but vary considerably in composition. The relationship between fat composition and plastid development is discussed.

INTRODUCTION

DURING a study of fat breakdown in germinating *Citrullus* (Crombie and Comber, 1956) it was noticed that as the cotyledons expand and turn green a new fat, characterized by a high content of linolenic acid (non-conjugated octadecatrienoic and octadecadienoic acids are termed linolenic and linoleic acids although the positions of unsaturation are unknown) replaces the original storage oil. Unexpanded, etiolated cotyledons at a comparable stage contain a much lower concentration of this acid (Hardman and Crombie, 1958). A similar synthesis of linolenic acid accompanies expansion and greening of young oil-palm leaves (Boatman and Crombie, 1958) and leaf fats from a number of sources are known to contain a high proportion of this acid (Meara, 1957).

It seemed probable that synthesis of highly unsaturated fatty acids was closely associated with chloroplast production, since chloroplasts have a relatively high lipid content (Menke and Jacob, 1941; Rabinowitch, 1945) and high iodine values for chloroplast and 'chloroplastin' fatty acids have been recorded (Menke and Jacob, 1941; Zirm *et al.*, 1955). The fatty acid composition of isolated chloroplasts and of green and non-green leaves (from etiolated and variegated plants) has therefore been examined in order to obtain further information on leaf fats.

MATERIALS AND METHODS

Leaf samples were weighed, cut into small pieces and dropped immediately into boiling ethanol. The solvent was removed under nitrogen at low temperature and the residue dried to constant weight at 80° C. Lipids were

extracted from the finely ground material with light petroleum (b.p. 40–60°) in a Soxhlet apparatus (8 hrs.).

Chloroplasts were isolated (at Oxford) by a method developed by James and Das (1957); leaf portions were homogenized (90 secs.) in about 150 ml. of medium (M./15 phosphate buffer; pH 7·1 containing 0·35 M. sucrose) in a Waring blendor. The homogenate was filtered through a double thickness of muslin, centrifuged (1 min. at $600 \times g$) to remove cell debris, centrifuged at higher speed (10 min. at $1000 \times g$) to throw down whole chloroplasts and the latter washed with fresh medium. All operations were carried out at a low temperature. The chloroplast precipitate was immersed in boiling ethanol, cooled and the lipids extracted by grinding alternately with ethanol and ethyl ether until the residue was colourless. The solvents were evaporated under nitrogen at low temperature and lipid material separated into light petroleum (b.p. 40–60°).

Chlorophyll was estimated on a portion of the original chloroplast precipitate by the method of Mackinney, 1941.

Analysis of the lipid fractions was carried out by methods already described (Boatman and Crombie, 1958).

RESULTS

1. *Fatty acids of etiolated and green* Vicia faba *leaves*

Three different stages in leaf development were examined; unexpanded etiolated leaves, slightly expanded green leaves, and fully expanded normal green leaves. It was found (Table I) that fatty acid content increases from 13·5 μg./leaflet in the etiolated sample to 26·7 μg. in the slightly expanded green leaf and 4,070 μg. in the fully expanded leaf. If, however, the results are expressed as mg. fatty acid/g. dry weight the relative figures are 12·3, 9·0, and 8·5, indicating a somewhat higher concentration of fatty acids in non-green leaves.

Microscopic examination showed that the majority of cells in the unexpanded leaves (both etiolated and green) were unvacuolated and contained no clearly distinguishable plastids. Microchemical investigation revealed the presence of plastid-like bodies, which stained black with osmic acid, located in numerous epidermal hairs; there was slight general staining of the protoplasm with osmic acid, but no evidence of fat accumulation in any other part of the leaf. In fully expanded leaves such epidermal hairs form only a small fraction of the total leaf volume.

Fatty acid analysis indicated the presence in all samples of palmitic (C_{16}), stearic (C_{18}), and long-chain saturated ($> C_{18}$) fatty acids. At least four unsaturated acids were also present corresponding, from their elution behaviour, to octadecatrienoic, octadecadienoic, octadecenoic (assumed to be oleic acid), and long-chain ($> C_{18}$) unsaturated acids.

Figures for the quantities of these acids were obtained by comparing chromatograms before and after removal of unsaturated acids by permanganate oxidation, but this method is incapable of detecting more than one

Table I

Fatty acids in leaves and chloroplasts

(*Figures in parentheses are octadecadienoic acid determined by UV*)

	Vicia faba				*Acer negundo*			*Zea mais*		*Ilex aquifolium*		*Ligustrum ovatifolium*	
	Etiolated	Small green	Large green	Chloroplast	White	Green	Chloroplast	White	Green	White	Green	Yellow	Green
Dry wt. as % fresh wt.	17·7	20·0	11·2	—	18·1	29·8	—	13·7	17·0	25·6	43·2	18·6	32·6
Lipid as % dry wt.	3·1	2·0	2·0	—	2·2	4·3	—	2·3	6·1	1·0	2·0	1·1	1·0
Non-saponifiable as % lipid	5·6	0·8	2·3	2·0	34·8	32·8	0·8	6·0	6·9	46·1	56·1	9·0	4·5
Total fatty acid as % lipid (wt.)	50·9	71·0	69·7	43·9	38·8	38·5	37·9	54·2	50·1	40·2	38·7	65·3	65·7
Fatty acid (determined chromatographically) as % lipid	39·6	45·6	43·2	29·2	25·7	31·6	20·2	29·8	34·4	19·4	19·7	25·6	32·2
Fatty acids as % chromatographically determined acid													
Myristic	—	—	—	—	1·0	0·6	2·4	—	—	—	—	1·5	1·3
Palmitic	16·7	11·7	7·1	7·4	12·9	11·0	12·6	13·4	8·0	17·8	20·5	14·8	11·8
Stearic	4·7	3·2	1·4	1·2	3·2	1·0	2·4	2·8	1·8	7·4	3·2	4·7	2·7
Long-chain satd. acids (as C_{22})	4·6	4·0	4·4	1·2	29·2	5·2	—	6·6	2·1	13·3	3·8	2·5	3·8
Oleic	0·0	3·4	4·6	5·2	11·7	6·8	10·0	10·1	6·9	11·5	14·1	14·7	20·5
Hexadecenoic	33·5	21·2	19·8	11·8	6·5	3·9	11·0	0·9	2·2	16·1	14·0	11·3	12·2
Octadecadienoic	(34·7)	(14·3)	(9·7)	(2·6)	10·1	11·4	(3·8)	36·2	7·0	(15·6)	(10·7)	(6·6)	(6·4)
Octadecatrienoic	39·4	56·0	61·1	72·0	15·9	51·5	61·1	27·3	69·0	20·7	23·7	39·0	43·3
Eicosenoic					2·5	2·1		2·7	1·4			2·2	0·3
Long-chain unsatd. acids (as docosenoic)	1·0	0·4	1·6	1·4	7·0	6·5	0·5	—	1·7	5·3	0·8	1·3	4·0

unsaturated component in a single chromatographic peak. However, since independent UV measurements of octadecatrienoic acid content agree fairly well with figures for the same acid obtained chromatographically, it is assumed that there is little, if any, other acid in this peak. Agreement between chromatographic and UV results for octadecadienoic acid, however, is poor, indicating the presence of more than one unsaturated component in this chromatographic peak (part of the discrepancy was due to a yellow pigment, but another unsaturated acid such as hexadecenoic acid may be present). Spectroscopic examination (Table II) showed that, although most of the triene was non-conjugated (λ_{max} after alkali isomerization 268 mμ), a very small proportion ($< 0{\cdot}5$ per cent.) was naturally conjugated (λ_{max} 270–4 mμ without isomerization). Part of the diene acid was also conjugated. There was no very definite evidence to suggest the presence of tetraenes, either conjugated or non-conjugated, although slight maxima occurred in the appropriate region in the large green leaves and in the etiolated leaves.

TABLE II

Examination of leaf fatty acids for conjugated material

Material	Diene region	Triene region	Tetraene region
Bean-etiolated	230–232 (11·3) m.	270 (5·0) m., 280 s.i.	315 (2·5) m., 300 s.i.
,, -small green	233 (21·2) m.	270 (13·7) m., 280 s.i.	—
,, -large green	233 (27·1) m.	271 (24·0) m., 263 and 278 s.i.	316 s.i.
,, -chloroplast	233 (20·6) m.	274 (12·3) m., 264 and 282 s.i.	—
Maple-white	230 (15·3)*	—	—
,, -green	230 (30·0)*	—	—
,, -chloroplast	230 (25·4)*	268–280 (17·3) m.	316 (12·3) m., 300 (13·1) m.
Maize-white	230 (15·5)*	274 (7·5) m., 285 i.	300 (5·5) m.
,, -green	230 (26·1)*	272 (17·7) m., 264 and 285 i.	300 i., 320 (11·5) m.
Holly-white	230 (28·0)*	268 s.i.	—
,, -green	230 (53·4)*	268 s.i.	315 (19·7) m.
Privet-yellow	230 (32·0)*	270 s.i.	—
,, -green	232 (37·6) m.	280–285 (24·6) m.	—

* Not a maximum: curve rising towards 220 mμ.

λ in mμ, figures in parentheses refer to absorption of a 1% solution in a 1 cm. cell (E_1^1)
m. = maximum, i. = inflexion, s.i. = slight inflexion

Although the same acids are present in the three different samples there are interesting differences in proportions. Etiolated leaves have a lower concentration of linolenic acid and a higher concentration of linoleic acid than either of the green samples which, despite the difference in size of the leaves from which they are derived, are very similar in fat composition. Extra fatty acid was synthesized during the development of chlorophyll in the young leaf (an extra 13·2 μg./leaflet on an original 13·5 μg. in the etiolated leaf). The percentage composition of this new fat was calculated and is as follows: linolenic 72, linoleic and other unsaturated acids eluting in the same position 9, oleic 7,

palmitic 7, stearic 2, longer-chain ($> C_{18}$) unsaturated acids 3. Clearly the fat formed at the same time as chlorophyll is highly unsaturated and is characterized by a high proportion of linolenic acid. It is interesting to speculate whether this unsaturated acid has any specific function in photosynthesis.

The association of linolenic acid with chlorophyllous tissue was confirmed by analysis of the lipid from isolated broad bean chloroplasts. The results are given in Table I and show great similarity with the calculated results above. Linoleic acid could not be detected spectrophotometrically but 3·8 per cent. of conjugated octadecadienoic acid was found (Table II). It is interesting that typical chloroplast fat should be synthesized at such an early stage in plastid development, although electron microscopy (Deken-Grenson, 1954) has shown that the undeveloped plastids in etiolated tissue undergo rapid and striking changes in structure when exposed to light. It is obvious from the fatty acid analysis of the large green leaves that there is a smaller amount of fat of different composition not associated with the chloroplast. This fat must contain most, if not all, of the non-conjugated octadecadienoic acid.

2. *The fatty acid composition of variegated leaves*

Three different plants with white leaf tissue were investigated—maple (*Acer negundo variegatum*), maize (*Zea mais japonica variegata*), and holly (*Ilex aquifolium*), and one plant with yellow leaf tissue—privet (*Ligustrum ovatifolium aureum*). Separate green and non-green leaves were used, except in maize where white and green portions were removed from the same leaves. Ethanolic extracts of the white leaves were faintly yellow-green and microscopic examination revealed that there were a few chloroplasts within the stomata; no plastids could be detected elsewhere. The extract from golden privet leaves was deep yellow (care was taken to select leaves exposed to direct sunlight which are less coloured than those in the shade and have no green tint). The yellow leaves contain some developed plastids.

White leaves were smaller and more delicate than the green and were characterized by a high fresh weight : dry-weight ratio (Table I). Starch grains which were abundant in the green leaves were absent from the white or yellow. Fatty acid content (per cent. dry wt.) was higher in the green leaves than in the white (maize 2·1 : 0·7, maple 1·3 : 0·5, holly 0·4 : 0·2), but in privet there was no difference in this respect between yellow and green leaves.

Fatty acid analyses for privet and holly followed the same procedure as those for the bean, but additional information was obtained in maple and maize by chromatography of the acids after hydrogenation. Results were calculated as before, except that components of the mixed octadecadienoic acid peak were estimated from the hydrogenation results (hexadecenoic from the increase in C_{16} acid after hydrogenation and octadecadienoic acid from the increase in C_{18} acid, after allowance had been made for the extra contribution made to this peak by oleic and linolenic acids): this resulted in good agreement of the results with those obtained spectroscopically. Conjugated diene acid was not certainly present (no λ_{max} at 233 mμ) in any of the samples except

green privet; in other cases there was strong absorption (but no maximum) in the diene region (Table II). Conjugated triene probably occurred in traces in all leaves except those of maple, and was not confined to green tissue; the ultra-violet maximum was well marked in maize with indications of the characteristic triple peak.[1] Traces of conjugated tetraene material may have been present in a few samples but the maxima were not well marked (Table II).

Differences in fatty acid composition between white and green tissues are most marked in maple and maize and these will be discussed first. The most striking effect is the high concentration of linolenic acid in the green leaves compared with the white (Table I), a result which recalls the similar association of this acid with green pigment in the broad bean. A second difference, particularly marked in maple, is the high concentration of long chain ($> C_{18}$) acids in the white leaves. In maize, linoleic acid is at a much higher concentration in the white parts, suggesting, as in *Vicia*, no correlation with the presence of chlorophyll. This effect was not confirmed in maple whose green and white leaves have similar concentrations of linoleic acid, but it was most interesting to find that isolated maple chloroplasts contained only traces of this acid.

Chloroplasts from bean and maple showed considerable similarity in fatty acid composition (Table I). Both preparations were contaminated with starch grains, but that from maple more than that from the bean: this is reflected in the chlorophyll-dry weight ratio after removal of alcohol and ether soluble material (1:39·6 in bean, 1:82·1 in maple). In both samples the ratio of chlorophyll: fatty acid (0·43:1 in bean, 0·36:1 in maple) was similar, but this is probably coincidental since it is known that the chlorophyll: lipid ratio can vary with season and age (Bot, 1942). There is also the possibility that some of the lipid found in the maple preparation may have been associated with starch grains (Sveshnikova, 1956).

In holly, unlike the two mesophytic leaves just discussed, differences in fatty acid composition between white and green leaves are slight, and in addition the total fatty acid content is low. Fatty acid analysis was complicated by the occurrence of a fairly high concentration of unidentified acidic material (7·9 and 19·9 per cent. respectively in white and green leaves, calculated for M. wt. 284) which eluted rapidly from the chromatographic columns: this material was destroyed by permanganate oxidation but was unaffected by hydrogenation. Since it was more plentiful in the green leaves it may be associated with the cuticle which was considerably thicker in these leaves than in the white ones.

Microscopic examination of green holly leaves revealed oil globules in the epidermal and adjoining cells and it seems probable that, in contrast to the mesophytic leaves examined, only a small proportion of the total lipid is

[1] Since the λ_{max} (272–4 mμ) is of longer wavelength than that for the *all-trans* material produced by isomerization (λ_{max} 268 mμ), it is unlikely that conjugation of linolenic acid has occurred during the mild saponification used to liberate the fatty acids. This point is also discussed by Zirm *et al.*, 1955.

associated with the chloroplasts. This may explain the similarity in fatty acid composition of white and green leaves of holly.

In privet the fatty acid composition of yellow and green leaves was similar, although the weight of fatty acid *per leaf* was greater in the green tissue (49·8 μg./leaf compared with 24·9). Linolenic acid was at a very slightly higher concentration in the green leaves but the differences in fatty acid composition are much less marked than those between albino and green leaves. It is interesting that yellow leaves also resemble green leaves in having a higher protein content than white leaves (Lakon, 1917).

DISCUSSION

The similarity in fatty acid composition of mesophytic green leaves (e.g. Meara, 1957, and the present analyses) is probably caused by a high proportion of their total fat being located in the chloroplasts (e.g. Menke and Jacob, 1941). Bean and maple chloroplast fats closely resemble each other and iodine value determinations on isolated spinach chloroplasts and on 'chloroplastin' fatty acids from a number of different leaves (Zirm *et al.*, 1955) indicate that these are similarly unsaturated.

Completely white leaves in which plastids are absent or poorly developed (e.g. maize, Randolph, 1922; maple, Funaoka, 1924 and Cramer, 1954, p. 355 and probably holly) have much less total fat than the green leaves and a much lower concentration of linolenic acid, the acid typical of chloroplasts. Long-chain ($> C_{18}$) acids and linoleic acid generally form a more important fraction of white leaf fat and this, taken in conjunction with their low proportion in chloroplast lipid, indicates that they occur outside the chloroplast. Yellow leaves in which some plastids were present (privet) resembled green leaves more closely, both in total fat content and in fatty acid composition. An appreciable quantity of linolenic acid was present, although less than in the corresponding green leaf. A similar result was obtained with yellow etiolated leaves of the broad bean, in which plastid-like bodies located in the epidermal hairs, were observed. It seems likely that well-developed plastids, even those from which chlorophyll is absent, contain an appreciable proportion of the total leaf fat and it is known (Sveshnikova, 1956) that leucoplasts as well as chloroplasts are a site of fat synthesis. Some of the triene acid therefore appears to be associated with plastid structure rather than with the photosynthetic function. It is, however, interesting to speculate on the extent to which alteration of the metabolic environment, which must result from the onset of photosynthesis, influences the degree of unsaturation of plastid fat.

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The Absorption and Distribution of Strontium in Plants

I. PRELIMINARY STUDIES IN WATER CULTURE

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SUMMARY

The absorption and distribution of strontium in barley plants has been studied, in water culture, both in the presence and absence of calcium. Tracer methods have been employed.

When no other bivalent ions were present in the external solution the absorption of strontium was proportional to the concentration over a wide concentration range (10^{-6} to 10^{-1} m.equiv./l.). In the presence of calcium, however, absorption was reduced and appeared to be dependent on the total concentration of calcium plus strontium rather than on that of strontium alone. The translocation of strontium from the root to the shoot increased as the external concentration of both ions was raised. In plants of low initial calcium status the rate of translocation of strontium was markedly reduced, apparently because of its retention at or near the root surface.

Autoradiographs of plants treated with labelled strontium for 24 hours at different stages of growth and grown subsequently for varying periods of time in unlabelled nutrient solutions showed that very little redistribution of strontium occurred within the plant. Leaves which developed after treatment in the labelled solutions contained little or no strontium even when the concentration in the older leaves was very high. Strontium was initially retained in the nodes of the stems, but the greatest accumulation was later found to be in the leaves which had been growing most actively at the time of absorption.

INTRODUCTION

THE occurrence of small quantities of strontium in plants grown under natural conditions has been recognized for many years (Robinson *et al.*, 1917, Headden, 1921), and in an extensive study of the absorption of cations by plants, Collander (1941) concluded that strontium was absorbed at the same rate as calcium. Both theoretical and practical considerations have encouraged the re-examination of this question in the past decade. The production of radioactive isotopes of the two elements has made it possible to study their interaction in the mechanism of absorption with a greater precision than hitherto. Moreover, a new importance has been given to such information by the fact that the isotopes of strontium, Sr^{89} (half-life 54 days) and Sr^{90} (half-life 28 years), are among the products of atomic fission which are potentially most dangerous to man and animals. This is because strontium is readily

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incorporated in bone where it may be retained for long periods. The ratio of strontium to calcium in diet is a major factor determining skeletal retention, and particular interest therefore attaches to the comparative study of the movement of the two ions through the food chain: soil → plant → animal → man.

It is well established that considerable discrimination between strontium and calcium may occur with respect to their metabolism in animals. For example, the ratio of strontium to calcium in milk is 0·10 to 0·14 of that in the diet of cattle (Comar, Russell, and Wasserman, 1957). Evidence is, however, scanty regarding the relative rates of movement of the two ions within plants. Whereas Collander (1941) considered that they moved at similar rates, Menzel (1954) concluded from studies with plants grown in soil that strontium was transferred to the shoots more slowly than calcium. The data hitherto available have not permitted unequivocal conclusions to be drawn.

The investigation here reported was therefore undertaken with the dual purpose of studying the absorption processes in intact plants and of helping in the assessment of the quantities of Sr^{89} and Sr^{90} which could give rise to radiobiological hazards. The first objective was inseparable from the second; the results of *ad hoc* studies can provide a valid basis for generalizations only when the operative mechanisms are understood.

The marked difference in the energy in the β radiation of Sr^{89} (1·48 meV) and Ca^{45} (0·25 meV) makes it simple to employ a double labelling technique for comparing the movement of the two ions in the same experimental material. Before undertaking such work the preliminary studies here described were, however, considered desirable. Labelled strontium was alone used and attention was directed to two subjects:

(*a*) the absorption of strontium from dilute solutions and its distribution in intact plants in the presence and absence of calcium;

(*b*) the extent to which strontium which has been deposited in leaves is subsequently redistributed.

The evidence of Rediske and Selders (1953) suggests that strontium moves out of leaves to a negligible extent. Further information on this question was sought not only because of the paucity of present knowledge of the mechanism whereby ions are translocated within plants but also because the extent to which strontium is redistributed would be an important factor determining the consequence of the aerial contamination of plants with fission products.

EXPERIMENTAL METHODS

Barley (variety—Proctor) was used throughout the work. The methods for water culture and assay described by Martin and Russell (1950) and Russell and Martin (1953) were employed. Short experimental periods (e.g. 24 hours) were used for the reasons discussed by these authors. Except when otherwise stated, plants were grown prior to the experiments in a nutrient solution containing: Ca^{++} 3 m.eq./l., K^{+} 6, Na^{+} 2, Mg^{++} 3, NO_3^{-} 10, $H_2PO_4^{-}$ 1, and SO_4^{--} 3 m.eq./l., together with micronutrients Fe, Mn, and B.

Since it was found that a large fraction of the strontium in the roots after relatively short absorption periods (e.g. 24 hours) was in a readily exchangeable form, it was necessary to determine whether significant losses would occur if the roots were washed at the time of sampling. Two methods of removing culture solution from the surface of the roots were therefore examined; the roots were washed in running water for 2 seconds and blotted dry with filter paper, or the solution was removed by suction through sintered glass plates sealed into glass funnels. No significant difference was observed and it was concluded that the rapid washing caused no significant loss.

In the early stages of the work Sr^{90} was employed as a tracer; subsequently Sr^{89} became available in high specific activities and was preferred because of its shorter half-life. When the two isotopes were used in parallel, similar results were obtained.

In some experiments autoradiographs were prepared to show the distribution of Sr^{89} throughout the plants. Fresh tissue specimens were mounted on cotton wadding pads, which were covered with cellophane and backed with plywood. Waxed cotton threads were secured round these mounts to retain the tissues in position. The specimens were covered with cellophane and exposed to Ilford X-ray film at −20° C.

EXPERIMENTAL RESULTS

The relationship between the external concentration of strontium and its absorption by plants under different conditions of calcium supply.

In Experiment A barley plants at the third-leaf stage were treated for 24 hours with the following concentrations of strontium labelled with 2μC/l. Sr^{89}: 2×10^{-6}, 6×10^{-5}, 2×10^{-3}, 6×10^{-2}, and 2·0 m.eq./l. The calcium supply was varied in two ways: *Pretreatment*. For 7 days prior to the experiment the plants were grown in the standard nutrient solution or in a calcium-free solution in which the 3 m.eq./l. Ca was replaced by 2 m.eq. K+1 m.eq. Na per litre. *Treatment*. With and without 2 m.eq. Ca/l. The treatments and pretreatments were combined factorially in five-fold replication. However, as the A.R. calcium chloride used as a source of calcium contained 0·02 per cent. strontium, the lowest concentration of strontium obtained in the presence of 2 m.eq./l. Ca was $1\cdot8\times10^{-4}$ m.eq./l.

Pretreatment with calcium complicated the interpretation of the results since it caused a marked effect on dry weight (Table I). Variations in the concentration of calcium or strontium supplied during the 24-hour treatment period, however, induced no significant changes. The very wide concentration range makes a logarithmic scale convenient for the comparison of the concentration of strontium in the plants (Fig. 1). Small differences are obscured by this presentation but a number of general trends are apparent.

The effect of calcium on the strontium content of both roots and shoots was similar over the four lower concentrations of strontium; the content of the roots and shoots bore an approximately linear relationship to the

external concentration and the application of calcium in the treatment period depressed absorption. Pretreatment without calcium had no effect on the concentration of strontium in the roots but it markedly reduced the concentration in the shoots. At the highest concentration of strontium different effects were observed. The presence of calcium in the treatment solutions now had very little effect on the concentration of strontium in the roots or shoots. Pretreatment without calcium, however, still reduced the concentration in shoots.

TABLE I

Experiment A: The effect on the dry weight of young barley plants of pretreatment in different nutrient solutions

Pretreatment m.eq./l. Ca	Treatment	Dry weight, mg.		Shoot as % total plant
		Root	Shoot	
0	0	35·0	115·6	76·8
0	2	36·8	117·8	76·2
2	0	53·7	133·4	71·3
2	2	55·1	135·5	71·1
	Sig. diff. ($P = 0.05$)	4·2	10·6	4·2

Some aspects of the relationship between the external concentration and absorption can be more clearly seen by examining the percentage of the applied strontium which was absorbed (Fig. 2). Since no dry-weight differences were induced by variation in the external concentration of calcium or strontium during the treatment period the shapes of these curves can be validly compared. When no calcium was applied in the treatment period the percentage of strontium absorbed was inversely related to the external concentration over almost the entire concentration range. In contrast the simultaneous application of calcium caused the values for the percentage absorption of strontium to remain constant when the external concentration was 6×10^{-2} m.eq./l. or lower; as 2 m.eq./l. of calcium were present the total external concentration of the two ions was virtually constant over this range. The change in the shape of the absorption curve induced by calcium therefore suggests that the *total* concentration of calcium and strontium rather than that of strontium alone was the main factor determining its absorption.

An examination of the manner in which strontium is distributed between roots and shoots shows, however, that the total concentration of strontium and calcium does not alone determine this process (Fig. 3). The percentage of the absorbed strontium which was recovered from the shoots (the transport index) was influenced both by the concentration of calcium in the pre- or simultaneous treatment periods and by the concentration of strontium. The highest values were obtained when calcium was applied in both periods and the lowest when no calcium was added. The influence of strontium, independent of calcium, is indicated by the fact that the transport index fell with decreasing external concentrations of strontium even when the total

concentration of strontium and calcium remained constant (i.e. in the 2×10^{-3} m.eq./l. strontium treatment in the presence of 2 m.eq./l. calcium).

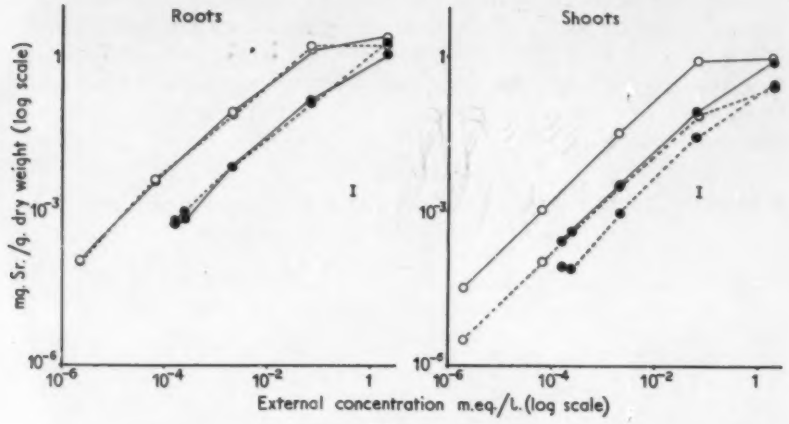


FIG. 1

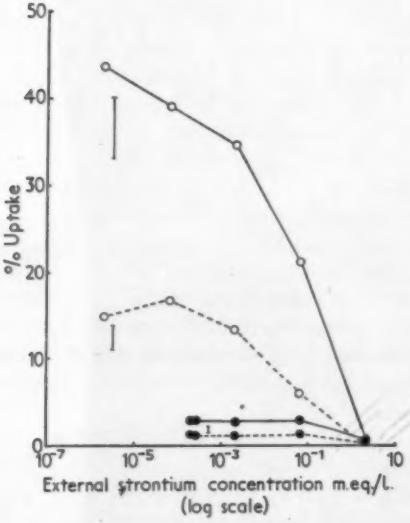


FIG. 2

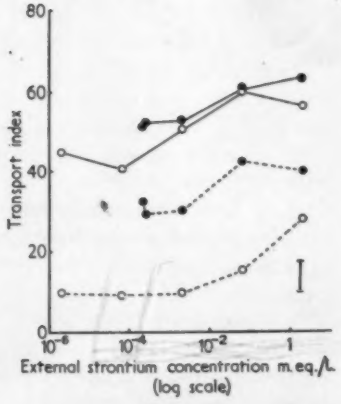


FIG. 3

FIGS. 1–3. *Experiment A*: The effect of the concentration of strontium in the external solution on (1) the concentration of strontium in the roots and shoots of barley, (2) the percentage uptake of Sr^{89} by barley plants, and (3) the ratio of shoot content to total plant content (transport index).

Solid line: plants pretreated with calcium; broken line: plants pretreated without calcium. Solid circles: 2 m.eq./l. calcium in experimental period; open circles: no calcium. 5 per cent. significant difference is shown.

THE REDISTRIBUTION OF ABSORBED STRONTIUM

In a preliminary experiment to obtain information on the redistribution of strontium, young barley plants at the second-leaf stage were treated for 24 hours in solutions containing 2 or 2×10^{-3} m.eq./l. of strontium labelled with 5 μc Sr^{89} per litre (Experiment B). Five replicates of each treatment were then sampled and 25 were transferred to normal nutrient solutions. Further sampling was carried out after 7, 14, 21, 28, and 35 days. The concentration of strontium did not affect plant dry weight but during the course of the experiment the dry weights of roots and shoots increased by factors of 2·6 and 6·0 respectively.

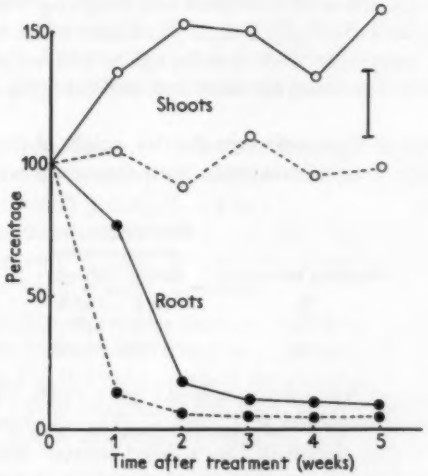


FIG. 4. *Experiment B*: The effect of time on the Sr^{89} content of the roots and shoots of barley plants treated for 24 hours in solutions containing 0·002 m.eq./l. (broken line) and 2·0 m.eq./l. (solid line) of labelled strontium and subsequently transferred to tracer-free nutrient solutions and sampled at weekly intervals. The Sr^{89} content is expressed as a percentage of that present in the roots and shoots of the plants immediately after treatment. 5 per cent. significant difference for shoots is shown.

Plants treated with the lower concentration of strontium showed constant values for shoot content throughout the experiment (Fig. 4); at the higher level of strontium the values increased, especially during the first 7 days, and the final value was approximately 50 per cent. greater than that at the initial sampling. The content of the roots decreased markedly with time, the final values for the high and low strontium treatments being 8·9 and 4·6 per cent. respectively of the initial values. In the first 7 days a considerably greater loss occurred from the plants treated with the lower level of strontium.

The contrasting trends in the content of roots and shoots shown in Fig. 4 suggests that equilibration did not occur between the strontium in the shoots

and that in the roots. Upward translocation therefore appeared to be an irreversible process. This question was further examined in Experiment C, in which three treatment periods of 24 hours, separated in time by 7 days, and three sampling occasions were employed. At the beginning of the experiment the plants were 28 days old and the programme of the experiment was as follows: at 28 days first treatment (T_1), at 29 days first sampling (S_1), at 35 days second treatment (T_2), at 36 days second sampling (S_2), at 42 days third treatment (T_3), and at 43 days third sampling (S_3).

The concentration of strontium applied in the treatment periods was 2×10^{-3} m.eq./l. labelled with 10 μC Sr^{89} per litre, and sufficient replicates were treated each time for six to be sampled on each subsequent sampling occasion. The following combinations of treatment and sampling occasions were thus provided: T_1S_1, T_1S_2, T_1S_3, T_2S_2, T_2S_3, T_3S_3. Plants which were not sampled immediately after treatment were transferred to tracer-free nutrient solutions. Five replicates were used for assay and autoradiographs were prepared from the sixth.

During the course of the experiment the dry weight of the plants increased considerably but the time of treatment with strontium was without effect. Mean values were:

	Dry weight, mg.	
Sampling occasions	Roots	Shoots
S_1	42·5	148·6
S_2	97·2	338·4
S_3	156·2	573·9

The results are shown diagrammatically in Figs. 5 and 6. The distribution of the labelled strontium absorbed at T_1 and T_2 (Fig. 5) changed with time in a manner similar to that shown in Fig. 4. Root content decreased markedly, a relatively small increase occurred in shoot content, and there were considerable losses to the external solution. Fig. 6 shows that whereas the concentration in roots initially exceeded that in shoots this relationship was rapidly reversed.

Autoradiographs of plants from the T_1S_1, T_3S_3, and T_1S_3 series are shown in Figs. 9*a–c*. A comparison of T_1S_1 and T_3S_3 (Figs. 9*a* and *b*) indicates that the time of absorption affected the manner in which strontium was distributed between leaves at the end of a 24-hour absorption period. Whereas the laminae of leaves 1, 2, and 3 contained a relatively uniform concentration of Sr^{89} in the T_1S_1 plant, the oldest leaves showed a relatively low concentration at T_3S_3. The midribs and leaf bases of actively growing leaves, however, contained a higher concentration than the laminae. It appears therefore that accumulation occurred mainly in the developing leaves, although some strontium was present in all the active expanded tissues. The lack of redistribution of strontium between leaves is clearly demonstrated by a comparison of the T_1S_1 and T_1S_3 treatments (Figs. 9*a* and *c*). In the interval between the two sampling occasions leaf 4 expanded and leaves 5 and 6 and three tillers

emerged. The activity in the shoot, however, remained almost entirely in the leaves 1–3, which had been present at S_1, and in the apical section of leaf 4, which was then expanding. The non-uniform distribution in this leaf suggests that the downward migration of strontium was slight even within individual leaves. A very light autoradiograph shown by leaf 5, barely visible in the reproduction, is attributed to strontium which was translocated from the roots or stems between S_1 and S_3; Fig. 5 provides evidence that some strontium was transferred from the roots during this period.

The redistribution of strontium in barley plants during the later stage of growth was examined in a subsequent experiment (Experiment D). The design was similar to that of Experiment C but the intervals between treatments were extended. The plants were 7 weeks old at T_1, T_2 was 2 weeks after T_1, when the flag leaf was expanding, and T_3 was another 5 weeks later, by which time the ears had emerged. All plants were of high calcium status at the beginning of the experiment but thereafter two levels of calcium were maintained:

High calcium plants—grown before and after treatment in normal nutrient solution containing 3 m.eq./l. calcium: treatment solution—10 μC/l. 'carrier-free' Sr^{89} with 3 m.eq./l. calcium.

Low calcium plants—grown before and after treatment in nutrient solution containing only 1 m.eq./l. calcium: treatment solution—10 μC/l. 'carrier-free' Sr^{89} with 3 m.eq./l. sodium.

In this way a steadily increasing calcium deficiency was developed in the low-calcium series. Calcium status had no significant effect on the dry weight of the roots (Table II), but the weights of the shoots of the low-calcium plants were significantly less than those of the high-calcium plants. Four replicates were used for assay and autoradiographs were prepared from the fifth.

TABLE II

Experiment D: The effect of time and calcium status on the dry weight of barley plants (g./2 plants)

Logarithmically transformed values are shown in italics

Age of plants (days)	Time of treatment and sampling	Roots				Shoots			
		Low Ca		High Ca		Low Ca		High Ca	
51	T_1S_1	0·85	*0·93*	0·88	*0·94*	3·50	*1·54*	3·92	*1·59*
64	T_1S_2	1·38	*1·14*	1·29	*1·10*	6·95	*1·84*	7·59	*1·88*
64	T_2S_2	1·28	*1·10*	1·21	*1·08*	5·86	*1·76*	6·97	*1·84*
101	T_1S_3	2·58	*1·41*	2·50	*1·40*	16·58	*2·22*	18·58	*2·27*
101	T_2S_3	2·51	*1·40*	2·73	*1·43*	14·11	*2·14*	17·42	*2·24*
101	T_3S_3	2·38	*1·38*	2·62	*1·42*	13·95	*2·14*	17·59	*2·24*
Sig. diff. ($P = 0·05$)			*0·12*		*0·12*		*0·08*		*0·08*

In Fig. 7 the content of roots and shoots and the extent of the subsequent loss of labelled strontium are expressed as percentages of the Sr^{89} initially absorbed. An inaccuracy in the results of the T_1S_2 series was introduced by

the fact that many of the older leaves died subsequent to treatment and some loss of fragments could not be prevented; this resulted in the loss of strontium from the shoots between T_1S_2 and T_1S_3. This apart, the pattern of dis-

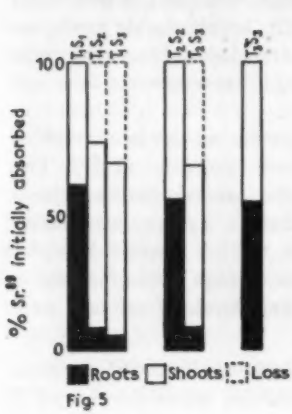


Fig. 5

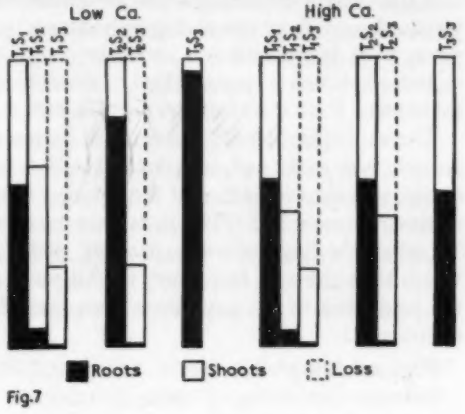


Fig. 7

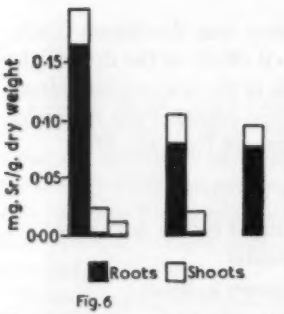


Fig. 6

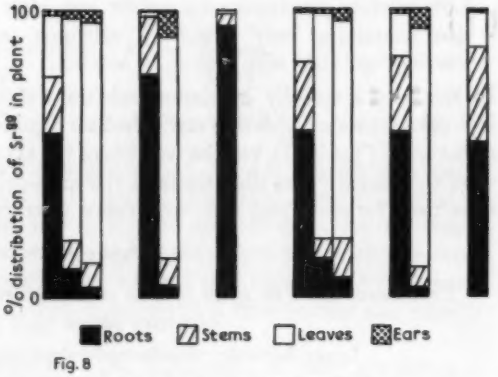


Fig. 8

FIGS. 5–8. FIG. 5. *Experiment C*: The percentage distribution of Sr^{89} between different tissues of barley plants. FIG. 6. The concentration of strontium in the roots and shoots of barley plants sampled at weekly intervals after treatment for 24 hours in a solution containing 0·002 m.eq./l. of labelled strontium. Details of treatment (*T*) and sampling (*S*) times are given in the text. FIGS. 7 and 8. *Experiment D*: The percentage distribution of Sr^{89} between different tissues of barley plants sampled at varying intervals after treatment for 24 hours in solutions containing 'carrier-free' Sr^{89}. Details of treatment (*T*) and sampling (*S*) times are given in the text.

tribution was qualitatively similar to that found in the two preceding experiments though the extent to which strontium was lost from plant roots tended to be greater. Plants which were treated after calcium deficiency had developed and were sampled immediately after the treatment with Sr^{89}, i.e. T_2S_2 and T_3S_3 (but not T_1S_1), showed a marked reduction in the fraction of the absorbed

strontium which reached the shoots (Table III). This result is in agreement with that obtained in Experiment A (Fig. 3). Concurrently with the reduction in the upward movement of strontium induced by calcium deficiency an increase occurred in the extent to which strontium was lost from the roots (compare T_2S_3 for high and low calcium, Fig. 7).

TABLE III

Experiment D: The effect of the calcium status of barley plants on the transport index (shoot content × 100/plant content) for strontium

Logarithmically transformed values are shown in italics

Time of treatment and sampling	High Ca	Low Ca	
T_1S_1	42·0	43·0	*1·62*
T_2S_2	41·7	18·8	*1·20*
T_3S_3	47·0	4·8	*0·65*
Sig. diff. ($P = 0·05$)	N.S.	..	*0·33*

In Fig. 8 the quantities of labelled strontium found in roots, stems, leaves, and ears are expressed as percentages of the total plant content. The highest values in ears at the final sampling occasion (S_3) were attained in plants treated at T_2—6·8±1·6 and 9·8±2·7 per cent. for high- and low-calcium plants respectively; the corresponding values for those treated at T_1 were 3·8±2·6 and 4·4±2·5 per cent. Only the high-calcium plants treated on the final occasion (T_3) showed any significant movement of strontium to the ears. The percentage of the total plant strontium which remained in the stems decreased with time except between T_1S_2 and T_1S_3 in the high-calcium plants. The increase here observed was, however, due to the decreasing total plant content (see Fig. 7) and not to an increased quantity of strontium in the stems.

Since some differences in weight occurred between the high- and low-calcium series (Table II) the ratio of the concentration of labelled strontium in the plants of the low-calcium series to that in the high-calcium series is a convenient basis for assessing the effects of calcium. In Table IV the data are presented in this form. As all the plants were of the same calcium status prior to T_1 the differences between the high- and low-calcium series at this time were due solely to the calcium applied to the high-calcium series during the treatment period. Table IV shows that the simultaneous application of calcium with strontium at T_1 reduced the concentration of strontium in all tissues—a result accordant with that obtained in Experiment A. This reduction was initially greatest in the ears and with the passage of time the difference between the two treatments decreased in roots and stems. Plants which had been treated after calcium deficiency had developed (i.e. at T_2 and T_3) showed strikingly different results. The concentration of strontium in leaves and ears (but not roots and stems) was now *lower* in the low-calcium series (T_2S_2 and

T_3S_3). This effect was transient only (compare T_2S_2 and T_2S_3). A further feature of the plants treated at T_3 was that the concentration in the stems was barely affected by the calcium status of the plants but the difference in the root concentrations was greater than at T_1.

TABLE IV

Experiment D: The effect of the calcium status of barley plants on the concentration of Sr^{89} *in different tissues*

Time of treatment and sampling	Ratio $\frac{\mu C\ Sr^{89}/g.\ in\ low\text{-}calcium\ plants}{\mu C\ Sr^{89}/g.\ in\ high\text{-}calcium\ plants}$			
	Root	Stem	Leaves	Ears
T_1S_1	7·0	10·5	7·0	14·5
T_1S_2	7·9	10·5	10·5	9·2
T_1S_3	3·4	4·6	8·8	10·9
T_2S_2	7·9	5·4	0·5	0·4
T_2S_3	7·2	7·6	4·2	5·4
T_3S_3	19·5	1·2	0·6	0·7

No great difference in the pattern of distribution of strontium in the shoots of the high- and low-calcium plants was apparent from the autoradiographs. The most striking feature in the plants examined immediately after the absorption of Sr^{89} (i.e. T_1S_1, T_2S_2, and T_3S_3) was the high accumulation of strontium in the stem nodes. On the earliest occasion accumulation was also marked in the ear primordia (Fig. 9*d*). With the passage of time (i.e. at T_1S_3 and T_2S_3) the accumulation in the nodes had decreased with a corresponding increase in the laminae of actively growing leaves (Fig. 9*e*). The amount of strontium deposited in the ears was considerably less than that in the laminae and appeared to be mainly in the rachis and the awns. Small patches of high activity on the leaves corresponded to sites of fungal attack.

DISCUSSION

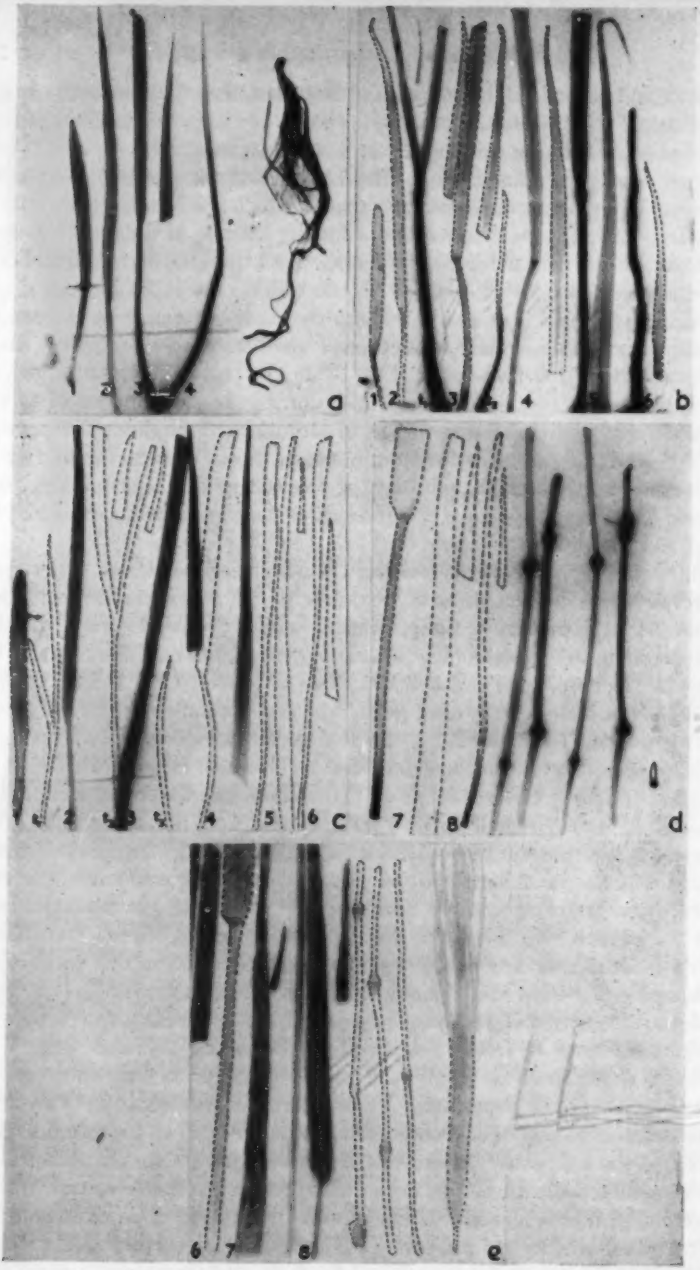
It is widely accepted that the initial entry of cations into roots of plants from dilute solutions is dependent on their exchange with mobile cations which are restrained by immobile anions at or near the root surface (Hope and Robertson, 1956; Middleton and Russell, 1957). Subsequently active transport occurs across the root and may be followed by upward translocation to the shoot. The marked loss of strontium which occurred when plants which had absorbed labelled strontium for 24 hours were transferred to normal nutrient solutions indicates that a large fraction of the strontium absorbed in this period was held in this superficial exchange mechanism. The quantity of strontium retained in the roots was little affected by their initial calcium content despite the fact that calcium simultaneously applied at a higher concentration caused a considerable reduction (Fig. 1). The magnitude of the fraction of the strontium in the roots which was lost to the external solution when plants were transferred to normal nutrient solutions was little affected by calcium, though there is some evidence (Fig. 4) that the loss was initially

more rapid from the plants containing less strontium. These results suggest that little, if any, strontium enters the root in exchange for calcium ions; an efflux of hydrions appears more likely to mediate its entry.

There is evidence that calcium affects the upward movement of strontium to shoots in a manner different from that in which it affects its entry into the root. In plants of low-calcium status a smaller fraction of the absorbed strontium was initially transferred to the shoots. Thus the addition of calcium to the external medium may cause either of two effects; the shoot content may be reduced by virtue of reduced entry into the roots, or it may be increased in consequence of a larger fraction of the absorbed material reaching the shoots. Both conditions are demonstrated in Table IV. In the T_1S_1 series, prior to which plants had received the same calcium supply, the addition of calcium markedly reduced the concentration of strontium throughout the plant. In the T_2S_2 and T_3S_3 series, in which the low-calcium plants had been deprived of calcium prior to the application of strontium, the high-calcium plants showed *higher* concentrations of strontium in their leaves and ears. This latter effect was, however, transitory.

Autoradiographs of plants nearing maturity (Fig. 9*d*) show that recently absorbed strontium was present in relatively high concentration in the stem nodes. With the passage of time strontium passed to the leaves and the nodal concentration was reduced (Fig. 9*e*). A suggestion that calcium interacts with strontium in its passage through the stems is provided by Table IV. When the high- and low-calcium treatments are compared after calcium deficiency had developed (T_2S_2 and T_3S_3), it is found that the effect of calcium on the concentration of strontium in stems was intermediate between that observed in roots and leaves. Since it is known that the vascular tissue is not continuous through the cereal node it appears possible that calcium and strontium may compete in their passage through the intervening tissue in a comparable manner to that which occurs in the initial phase of exchange entry into the roots. Equally the results suggest the occurrence of competition in the active transport mechanism, which leads to the release of ions into the vascular tissue in the roots. It is well established that the interaction of ions one with another is dependent on their ionic charge and mass (Middleton and Russell, 1957). A series of such reactions occurring in sequence would effect progressive partition, the ratio of calcium to strontium increasing with each step.

At first sight, however, this type of mechanism appears difficult to reconcile with the evidence of Experiment A that, when the concentration of strontium is low relative to that of calcium, the rate of absorption of strontium is determined by the total concentration of the two ions, so that strontium behaves as if it were effectively diluted by calcium and moving at the same rate. Such an effect could, however, result if the greater relative retention of strontium in the early stages of its upward passage is balanced by a corresponding reduced movement to the tissues most remote from the root. More detailed experiments must, however, be carried out before the adequacy of this interpretation can be established.



FIGS. 9 *a–e*. Autoradiographs of dissected shoots of barley plants, coleoptiles on left; leaf and tiller numbers are shown and when cut apical parts of leaves and stems are to the right of their bases.

Experiment C. 9*a* T_1S_1 *Experiment D.* 9*d* T_1S_1
9*b* T_2S_3 9*e* T_2S_3
9*c* T_1S_3

Details of treatment (T) and sampling (S) times are given in the text.

There is strong evidence from autoradiographic studies that strontium which has reached the leaves is translocated to other organs to an extent so small that it cannot readily be measured. The autoradiographs show that the maximal content is attained in the actively growing leaves, from which little migration occurs to subsequently formed tissues. The small quantity found in leaves which developed after the strontium had been absorbed can be explained in terms of release from the stems and continued upward movement from the roots. Downward translocation was not apparent even within individual leaves (Fig. 9*c*) and the content of the ears was very low (Fig. 9*e*). These results suggest that when absorption occurs continuously throughout the life of the plant the highest concentrations would be found in the oldest leaves. This accords with the evidence of Rediske and Selders (1953).

The manner in which strontium is distributed between leaves is readily compatible with the concept that transpiration is the major factor affecting the distribution of ions *after* they have been released into the vascular stele (Hoagland, 1944; Russell and Shorrocks, 1957). However, since strontium is markedly retained at each node in the stem, it is apparent that its distribution cannot reflect exactly the relative extent of transpiration by the different leaves. Furthermore the relatively high concentrations observed in ear primordia (Fig. 9*d*) which were ensheathed with leaves at the time of absorption is suggestive that metabolic processes which are as yet unidentified may play a part in the translocation of this ion. Spots of high activity on the laminae of old leaves (Figs. 9*c* and *e*) correspond with the sites of fungal attack. A similar high concentration of a wide range of substances on diseased areas of leaves has been reported by Yarwood and Jacobson (1955), who suggest that the accumulation may be due to the high metabolic activity of the pathogens. It would seem possible that an increased rate of water loss at the lesions may also be a significant factor.

ACKNOWLEDGEMENTS

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The Nuclear Incorporation of Iron and its Significance in Growth

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SUMMARY

Pea roots were grown in solutions containing low concentrations of Fe^{59} and using the technique of autoradiography the intracellular incorporation of the labelled iron into sections of these roots was examined. The Fe^{59} was incorporated into the nuclei to a much higher concentration than in the surrounding cytoplasm. This was the situation when the root tips were fixed in Zirkle's fixative, absolute ethanol, or in neutral formalin, but not when they were fixed in acetic alcohol. The iron which is incorporated into the nucleus can mostly be removed by treatment with dilute acids such as N. HCl.

It is concluded that normally iron is an important constituent of plant nuclei in addition to being a component of many essential enzymes. The importance of the present observations are discussed in relation to our earlier work which described the effects of iron deficiency on the growth of excised pea roots. It is suggested that the cessation of cell division in iron-deficient plants may be directly related to the observation that iron is a constituent of the nucleus.

INTRODUCTION

We have recently shown that when pea roots are cultured in a medium deficient in iron growth ceases abruptly and that this is a consequence of an abrupt cessation of division in the apical meristem (Brown and Possingham, 1957). The deficiency does not affect cell expansion, protein accumulation, and certain metabolic processes, all of which continue vigorously after division has ceased. When growth has ceased division is reactivated by supplying iron to the deficient medium. The evidence suggests that iron is immediately involved in the division process, and that when this ceases it is due to the exhaustion of a supply which is required in the meristem for the maintenance of division in that region.

In this context the intracellular distribution of iron in meristematic cells is a matter of immediate importance, and the results of this phase of the investigation are reported here. The subject has been investigated by growing seedling roots in a solution of radioactive iron and subsequently examining the distribution of the isotope by the autoradiograph technique of Doniach and Pelc (1950). In the design that has been used here the roots are attached to the seed from which they have germinated throughout the experimental period, and they are exposed to the solution containing the isotope for about 12 hours. Thus, the apical cells that are subsequently examined have been

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formed in conditions of vigorous growth and they have been formed in the solution. With this arrangement the distribution of the isotope is not a consequence of an exchange reaction with iron that is already present in the tissue, but is a result of incorporation into a tissue while it is being formed.

MATERIALS AND METHODS

The roots of 2-day-old pea seedlings var. Meteor were grown in aerated solutions containing 0·1 or 1·0 μc./ml. of Fe^{59} as Ferric chloride for periods of 10–15 hours at 25° C. The experimental solution was placed in a glass container covered with a perforated bakelite disk. The roots were threaded through the disk and into the solution. The disk was traversed by a tube which extended into the solution and through which air was circulated throughout the experimental period. The cultures were placed in an incubator and maintained in the dark.

After treatment the terminal 10·0 mm. tips were excised from the experimental roots. These were washed in several changes of distilled water and treated for 2 hours with one of the following: Zirkle's fixative (1929), absolute ethanol, acetic acid-ethanol (1:3) mixture, or neutral formalin (1–5 per cent. neutral formaldehyde in 1·0 per cent. $CaCl_2$). Following fixation the tips were washed in water, dehydrated through an alcohol series, cleared through a xylene series, and finally embedded in paraffin. Longitudinal sections usually 5·0 μ thick were cut using a Cambridge rocking microtome. The ribbons were attached to gelatin-treated slides. After removal of the paraffin the sections were washed in running water for approximately 1 hour. Finally, fine-grain stripping film (Kodak A.R. 10) was applied to the preparations, after which the slides were placed in a box covered with thick black paper to exclude light, and were transferred to a refrigerator in which they were stored for a period which varied between 10 and 30 days. The autoradiographs were developed and fixed in the solutions recommended by the manufacturers of the stripping film. Before being examined the sections were stained through the photographic film with the water-soluble Giemsa stain recommended by Lajtha (1954).

The final preparations were examined with a binocular microscope equipped with a ×50 fluorite oil-immersion lens. Representative slides were photographed with this assembly to which an eyepiece camera had been attached. Certain of the photographs obtained are reproduced in Plate I, and it will be noticed that these are not in focus with respect to cytological detail. This is a necessary consequence of the silver grains in the stripping film being at a higher optical level than the tissue section, and the photographs have been taken at an intermediate focal level which shows the grains against the general outlines of the cellular structures.

In one series of observations squashes instead of sections were used. These were prepared by the standard procedure of treating the tips after fixation with 1·0 N. HCl for 10 minutes at 60° C. and subsequently spreading the tissue into a single layer of cells by pressing under a thin plastic strip. These

preparations were made on gelatin-treated slides and the plastic strips subsequently floated off in water. The separated cells remained anchored to the gelatin, and the whole preparation was treated as were the sections for the development of the autoradiograph. In this series of observations the tissue was in certain instances fixed in acetic alcohol and in certain others in Zirkle's mixture.

Experiments were undertaken to explore the possibility of two types of artefact. The first that the local development of silver grains might be induced by the fixative itself, and the second that the fixative might promote a redistribution of iron within the cell. The first was examined by making preparations from tissue which had not been exposed to the isotope, and the second by immersing untreated tips in a fixative with solution which also contained the radioactive isotope. In neither case were local accumulations of the isotope found which were comparable with those observed when the original tissue had been formed in an experimental solution.

RESULTS

The results obtained with sections after treatment of the tissue with neutral fixatives are shown in Figs. 1–4.

Fig. 1 shows a longitudinal section from a root which had been grown for 12 hours in a solution containing Fe^{59} at a concentration of 0·1 μc./ml. and which had been fixed with Zirkle's fixative. Fig. 2 shows part of the same preparation but at a higher magnification.

Figs. 3 and 4 also show longitudinal sections from tips which had been exposed to the isotope at a concentration of 0·1 μc./ml. The tissue of Fig. 3, however, was fixed in absolute ethanol and that of Fig. 4 in neutral formalin.

It is clear that with the fixatives used the results indicate a greater accumulation of iron in the nucleus than elsewhere in the cell. Occasional silver grains may be seen above the cytoplasm but, undoubtedly, the greatest concentration is in the nucleus. Occasional preparations have suggested an accumulation of iron at the periphery of the nucleus, but the results have not been sufficiently decisive to indicate this as a definite conclusion.

The results shown have only been obtained with neutral fixatives. Whenever acid fixatives were used or whenever acid treatment has been applied after fixation an accumulation of iron in the nucleus has not been observed. After fixation in acetic alcohol and treatment with normal hydrochloric acid in squashes a uniform distribution of the isotope was found. A similar result was obtained when sections from tissue fixed in Zirkle's fluid were subsequently treated with 1·0 N. HCl.

The results suggest that while iron is normally incorporated into the nucleus, it is held in this position in some form of combination which is highly acid labile.

It may be noted that none of the photographs show iron in mitotic figures. This is a feature of all the results obtained. Incorporation has been observed only with interphase nuclei.

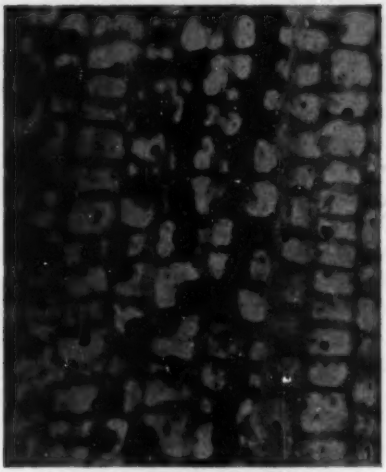

FIG. 1. Section from root treated with Zirkle's fixative (×500).

FIG. 2. Same as 1 but at higher magnification (×800).

DISCUSSION

It may be emphasized that the technique used here does not show the distribution of all the iron that is normally present in the cell. In particular it probably does not show the position of the free inorganic iron that is certainly present in large quantities. Most, if not all, of this fraction is certainly dispersed during the fixation and subsequent washing procedures that the

FIG. 3. Section from root treated with absolute ethanol (×500).

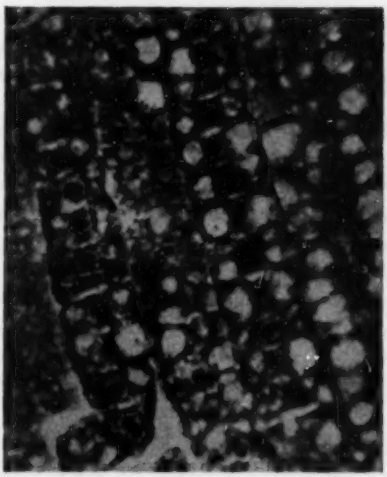

FIG. 4. Section from root treated with neutral formalin (×500).

technique involves. Further, it may be noted that since the isotopically labelled element is not the only iron available to the tissue, this certainly also contains non-radioactive iron. Iron is available to the root from reserves in the seed. This, however, is not likely to affect the distribution indicated by the labelled isotope since there is no reason to believe that the seed reserve is more readily incorporated into certain cellular structures than it is into others.

The general conclusion that has been reached in this investigation corresponds with results reported by earlier workers. Poulsen and Bowen (1952) found that Fe^{59} administered orally in *Drosophila* sp. is incorporated into the cells of the fat body and the salivary gland. It was observed that in these cells the concentration of Fe^{59} in the nucleus is 5–10 times greater than it is in the surrounding cytoplasm. Further, that the iron in the nucleus is in a form of combination which is resistant to neutral alcohol/formalin fixation and to fat solvents such as dioxane. Again Kirby (1956) has reported that the concentrations of iron and manganese in rat-liver nuclei isolated both in aqueous and non-aqueous media are higher than they are in whole liver.

In this connexion, results recently described by Kessler (1957) are of some significance. This investigator has found a close correlation between the content of DNA and that of soluble iron in the leaves of higher plants. It is significant that the soluble-iron fraction is that which is extractable with 1·0 N. HCl. It has also been shown that adenine and guauine sprays induce an increase in the level of DNA with a corresponding increase in the content of soluble iron. Kessler's results suggest a necessary connexion between iron and DNA; ours do not indicate this but since DNA is probably restricted to the nucleus they are clearly compatible with those of Kessler. They are also compatible with those of Kirby (1956) who finds that DNA is released from tissue extracts in the presence of chelating agents such as cupferon, potassium cyanide, and sodium azide. This worker has also found that the binding of DNA to collagen may be promoted by heavy metals such as iron, copper, and zinc.

Iron is recognized as being present in the cell as a component of a porphyrin in certain oxidizing enzymes. The acid-labile character of the iron complex identified here suggests that it is not involved in these, and indeed Dounce (1943) has shown that in isolated rat-liver nuclei catalase and cytochrome oxidase activity is both relatively and absolutely low. The present series of results, of course, do not in any way suggest that iron does not make an important contribution to metabolism by being incorporated into the oxidizing enzymes. The evidence, however, does suggest that a large fraction of the total iron content may be required in the formation of the nucleus and this conclusion is compatible with earlier results on the effect of iron deficiency on growth.

With pea roots grown in isolated culture it has been shown that with a deficient medium growth continues at an unrestricted rate until a stock of iron introduced with the innoculum is exhausted. At this stage cell division ceases abruptly, while other processes are apparently unaffected. After the cessation of division, cell expansion and accumulation of protein and respiration continue vigorously. Further observations along the length of the root immediately after division has stopped are consistent with the interpretation that the disturbance to growth is localized in the meristem and that immediately after growth has ceased the main body of the deficient root is similar to that of a normal root. In the meristem at the moment when growth ceases

respiration is normal and the deficiency seems to be affecting the division process directly. After division has ceased in the deficient roots the cells immediately behind the restricted but persistent apex expand laterally to form a subterminal bulb. A similar symptom has been recorded by Carlton (1954) in zinc-deficient roots in which division has ceased and in roots in which division has been arrested by toxic concentrations of naphthylacetic acid.

It is of some significance that a set of morphological changes similar to those observed in isolated roots have recently been recorded in intact tomato seedlings. The growth of seedlings in full and deficient media was compared. It was found that both continued to form primordia at the same rate until the induction of these ceased in the deficient series. Ten days after planting the controls carried nine foliar units and the deficient plants eight. After this stage, however, further primordia were not formed in the deficient series. The abrupt cessation in the formation of primordia suggests a correspondingly abrupt cessation in division. On the deficient seedlings the roots also developed the subterminal bulb observed in the isolated cultures.

With deficient seedlings the formation of primordia is resumed immediately iron is supplied, but during the period of deficiency and after growth has ceased the apex persists and remains indistinguishable from that of a normal plant. This is clearly consistent with the conclusion that the arrest of growth is due to the exhaustion of a supply of labile iron which is required in division, and that the main body of the plant is virtually metabolically intact after growth has ceased. It is also consistent with the results of Mason, Oldewurtel, and Propst (1952) who compared the activities of a series of enzyme systems in deficient and control plants. Only with respect to polyphenol oxidase and peroxidase were large differences recorded. The activities of ascorbic-acid oxidase, lactic-acid dehydrogenase, DPNH diaphorase, and glycollic-acid dehydrogenase were virtually the same when expressed on a unit protein basis and of respiration when expressed on a dry-weight basis. Our results with pea roots suggested that after the cessation of growth no further components of the cytochrome system were synthesized but that invertase protein continued to be elaborated.

Elsewhere we have suggested that the cessation of division may be due to the exhaustion of a supply of labile iron with a consequent failure in the synthesis of the cytochrome system. After growth has ceased the evidence suggests that although protein continues to be elaborated nevertheless the cyanide-sensitive component of respiration does not increase. If cytochrome is involved in division then the evidence is compatible with the view that cessation of division is a consequence of failure in the synthesis of cytochrome. On the other hand, the data do not exclude the alternative interpretation that the cessation of cytochrome synthesis is a consequence of the exhaustion of a supply of iron which has been consumed in some synthetic process which is necessary to division but which does not involve cytochrome. The present series of results, although they do not indicate the second alternative

interpretation, at least make it more plausible. In the terms of this, the position would be that under deficient conditions with each division iron is withdrawn from a labile pool and becomes incorporated in the newly formed nuclei and cytoplasmic components. It may be supposed that the nuclear requirement is greater than the cytoplasmic and that it is this which finally exhausts the labile supply. When a sufficient quantity of free iron is no longer available for the formation of the nucleus, growth and the formation of iron porphyrin enzymes necessarily also cease.

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Nitrogen Metabolism, Respiration, and Growth of Cultured Plant Tissue

PART IV. THE IMPACT OF GROWTH ON PROTEIN METABOLISM AND RESPIRATION OF CARROT TISSUE EXPLANTS. GENERAL DISCUSSION OF RESULTS

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SUMMARY

Critical examination of the amino-acid composition of proteins in fast-growing and slow-growing tissues reveals only very small differences, indicating that some factor other than the amino-acid complement is responsible for, or reflects, the great increase in the mass of protein in the fast-growing tissues.

Increases in fresh weight and total protein are exactly parallel, indicating that water uptake is an active process associated with growth. Respiration, on the other hand, increases far more in the fast-growing over the slow-growing tissues than does total protein. A given amount of protein in the fast-growing tissue will support a much greater respiration rate than the same amount in slow-growing tissue.

The incorporation of radioactivity into amino-acids of the protein indicates that there are two distinct types: those in which incorporation is increased in fast-growing tissue much more than the total protein, and to the same extent as respiration (notably glutamic acid, aspartic acid, and threonine); and those in which the increased incorporation is much smaller, slightly less than total protein (notably proline and hydroxyproline). It is concluded that there are two main protein fractions: the 'active' moiety, which is undergoing rapid breakdown and resynthesis, giving rise to much of the CO_2 through oxidation of its residues; and the 'inactive' moiety, which once synthesized is not reutilized or broken down. It is the former, or 'active' protein whose synthesis is greatly increased in the fast-growing tissues, and it is the pace, rather than the kind, of reactions which differentiates between the fast- and slow-growing tissues.

The entire experimental data are discussed with reference to a number of current theories and investigations. A number of experimental observations are noted which admit of interpretation along the lines here developed.

IN the first paper (Parts I, II, and III)[2] an investigation was described in which C^{14}-labelled substrates were supplied to carrot explants. C^{14}-glucose, C^{14}-γ-aminobutyric acid, and C^{14}-glutamine were supplied in trace amounts to slow-growing (i.e. without coconut milk) or fast-growing (i.e. with coconut

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[2] Parts I to III, this volume, pp. 11–51.

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milk) cultures, and the metabolic fates of these compounds were followed through the alcohol soluble and insoluble fractions of the cell and into respired carbon dioxide by chromatography and radioautography. Total and specific activities of carbon in various compounds were determined, and the apparent specific activity of the carbon in transit to or from the individual metabolites was calculated. All the experimental data will be found in Tables I–XVII of the earlier paper, and reference to these will be made in the text to follow.

The interpretation embodied three main ideas which are illustrated in Fig. 1, Part III, which is here reproduced (p. 291). These are (i) the cell is divided into phases or compartments; one associated with assimilative metabolism and protein synthesis; and others with protein breakdown, catabolic metabolism, and storage. (ii) There are two main sources of carbon dioxide

TABLE XVIII

Effects of the treatments (with and without coconut milk) on fresh wt. (mg./explant), cell number (thousands/explant) and average cell size (μg./cell)

Treatment	Weight	Cell number	Cell size
Slow-growing (no c.m.) at:			
7 days	5·1	23·5	0·217
12 days	7·6	30·9	0·244
Fast-growing (+c.m.) at:			
7 days	15·2	195·0	0·078
12 days	28·0	340·0	0·083

in the cell, one arising by the direct oxidation of sugars, and the other from the oxidation of breakdown products arising from protein turnover. (iii) Free amino-acids are not immediate precursors of protein. Protein is formed from carbon skeletons derived from glucose and nitrogen groups, which are combined and immediately converted to protein at a site remote from the soluble amino-acids.

In this section attention will be focused on the differences between the rapidly-growing tissue and the slowly-growing tissue, to discern whether these differences are of kind or degree.

Lastly, the new ideas here developed are related to other tissue systems currently under investigation.

Morphology of the tissue. Explants from dormant carrot roots, obtained in the manner described, may have a residual ability to grow in a medium unsupplemented by the coconut milk, but this varies with the strain of carrot and even with the particular root. In the experiments here presented the tissue explants without coconut milk did increase in fresh weight to some extent. However, the fast-growing tissue, subjected to the coconut-milk stimuli, grew very much more and contained per unit fresh weight a larger number of smaller cells, showing that the main effect, in the faster-growing tissue, is a much accentuated rate of cell division. Data from other experiments permit

an estimation of the number of cells in the original tissue explant, the number present at the end of the pre-period of growth, and the probable number in the tissue which had attained the final weight. These figures are shown in Table XVIII. Thus, the fast-growing tissue places the emphasis on cell division in contrast to the slow-growing tissue which is more concerned with cell enlargement.

Composition of the protein of the tissue during growth. The analytical data furnish the complete amino-acid composition of the alcohol-insoluble nitrogen fraction for the tissues under each experimental condition throughout the experimental period. Amino-acid analyses of the protein have been recalculated to show the molecular ratios of the various nitrogen constituents, arbitrarily designating alanine as 10.

TABLE XIX

Mean relative molar composition of proteins in carrot tissue explants, as affected by rate of growth, time, and the C^{14}-labelled compounds supplied. (*Based on Alanine* = 10)

(Explants had been cultured for 7 days at time 0, 9 days at time 2, and 12 days at time 5)

Type of tissue	Aspartic acid	Glutamic acid	Serine	Glycine	Threonine	Alanine	Valine	Leucines	Lysine	Arginine	Proline	Hydroxy-proline
Average of:												
All slow growing (no c.m.)	10·3	11·1	8·3	11·2	8·3	10·0	8·3	12·2	3·6	1·8	15·6	9·8
All fast growing (+c.m.)	10·2	11·2	7·2	12·8	7·4	10·0	7·2	11·2	5·6	3·1	12·7	4·5
Slow growing, time 0	8·7	12·5	7·9	10·1	8·8	10·0	8·0	12·2	3·7	2·4	15·6	11*
Slow growing, time 2	10·7	11·2	8·6	10·8	8·7	10·0	8·2	11·8	4·3	2·0	18·7	8
Slow growing, time 5	10·6	10·7	7·6	12·4	7·5	10·0	7·5	12·0	3·8	2·2	12·7	10
Fast growing, time 0	8·4	11·0	7·1	10·7	6·4	10·0	7·0	12·4	5·7	2·8	9·3	4
Fast growing, time 2	9·8	10·7	6·1	12·7	6·8	10·0	6·8	11·3	6·0	3·0	9·8	4
Fast growing, time 5	11·6	11·1	(5·9)	14·3	7·1	10·0	6·4	11·5	6·3	3·2	12·2	5
C^{14}-Glucose supplied	10·1	11·1	7·4	12·0	7·9	10·0	8·0	11·9	4·4	2·7	14·7	8
C^{14}-γ-aminobutyric acid supplied	10·1	11·0	8·2	11·7	7·7	10·0	7·6	12·3	5·1	2·8	13·4	8
C^{14}-Glutamine supplied	11·0	11·3	7·5	12·7	8·1	10·0	7·8	11·7	4·2	2·8	14·8	7

* Time 1

Note: Histidine and cysteic acid were found in all tissues; tyrosine and phenylalanine were found in most. The quantities of these substances were not measured.

To provide the most accurate basis for comparing the molar composition of the protein of rapidly-growing tissue with that of slow-growing tissue, all the data for each of these two categories have been pooled without regard to differences due to time or the C^{14}-compounds supplied. Similarly, in order to show most clearly the effects of time on either slow- or rapidly-growing tissue proteins, the data for various time intervals (periods 0, 2, and 5, representing tissue cultured either with or without coconut milk for 7, 9, and 12 days respectively) were pooled without regard to differences due to the C^{14}-compounds supplied. Again, in order to show any differences which might result from the supply of C^{14}-labelled glucose, γ-aminobutyric acid or glutamine, the data for all the samples supplied with each of these compounds were

bulked without regard to the effect of time or the conditions of growth. All these data, showing mean molar composition of the proteins in the tissue relative to a value of 10·0 for alanine, are presented in Table XIX. Similar calculations have not been made for the alcohol-soluble fraction, because there is no direct relationship between the relative amino-acid composition of the soluble fraction and the amino-acid composition of the protein.

All of the data in Table XIX represent the amino-acid composition of the protein in tissue which has undergone some growth. Even the tissue at the period designated Time 0 had departed from the condition in the intact organ by the effects of the pre-period of 7 days, spent either with or without coconut milk. The comparison of these two types of tissue at Time 0 therefore shows the effect of 7 days' culture, with or without coconut milk, during which some effect was already apparent on their fresh weights (see Table II).

The outstanding conclusion to be drawn from these data is that the composition of the protein is remarkably constant despite the very wide differences in the growth responses. This is evident from a general comparison of the protein analyses made on all the slow-growing tissues with those made on all the fast-growing tissues, and also shows from the data illustrating the effects of time and the C^{14}-compounds supplied on the composition of the protein. Therefore, the overall conclusion is that the composition of the protein is remarkably constant throughout the whole of these experimental treatments with only minor differences to be enumerated below. Thus, the great effects which were induced on the tissue by the experimental treatments must reflect the quantity or metabolic activity of the protein which it contained, rather than its kind.

The outstanding points of difference in the relative composition of the protein are as follows.

It has been observed previously that a characteristic of the tissues cultured on coconut milk containing media is that they contain hydroxyproline in greater amount in their protein than does tissue as it exists in the intact organ. This has been shown to be true for carrot tissue, for potato tuber tissue, and for certain plant tumors (Steward and Thompson, 1954; Steward, *et. al.* this volume, p. 1). It will be noted here that both fast- and slow-growing tissues contained relatively large amounts of proline and hydroxyproline, the slow-growing tissues, surprisingly, containing more than the fast-growing. However, the data for various time intervals show that even though proline and hydroxyproline were present in relatively larger amounts in the slow-growing tissues after 7 days' culture, their relative contents declined with time thereafter. In contrast, their relative amounts in the protein of fast-growing tissue increased with time during the experimental period.

Further comparison shows that the fast-growing tissue contained more glycine, lysine, and arginine, while the slow-growing tissue contained more serine, threonine, valine, and leucine(s) in addition to the prolines. The enrichment of the protein of the fast-growing tissue with basic amino-acids (arginine and lysine) is of interest, and is reasonable since these tissues might

well be richer in nucleoproteins, as indeed is the bulk of protein in plant-growing points (Steward, Wetmore, Thompson, and Nitsch, 1954).

The effects of time on the composition of the tissue proteins are not large. There is a tendency toward an increase with time in the proportions of aspartic acid and glycine in both slow- and fast-growing tissue. Glutamic acid decreases slightly with time in the slow-growing tissue; threonine is increased in the fast and decreased in the slow; the basic amino-acids increased with time in the fast-growing tissues. The prolines, as mentioned above, increase in the protein of the fast and decrease in that of the slow-growing tissues.

The data on the effects of supplying the tissue with the various C^{14}-labelled compounds, show that the differences which occur in the composition of the proteins are small and random. There appears to be no consistent effect of the supply of nitrogen compounds as compared to the supply of carbohydrate, in spite of the large response which occurred in the soluble nitrogen. It is probable that the quantities of the compounds supplied were too small for their effect to be felt in the relatively far removed process of protein synthesis. This point is mentioned in justification of the use of small 'tracer' amounts of labelled metabolites which, although they may cause considerable unbalance in the reactions involving soluble compounds, exert no influence on such a basic part of cellular metabolism as protein synthesis.

It has been shown (Roberts *et al*, 1955) that the Krebs cycle and synthetic reactions of *Escherichia coli* are very finely balanced and that even small amounts of exogenously added metabolites tend to upset this balance, causing 'leakage' of intermediates into the medium. In the present experiments, although a limited amount of unbalance was produced in the soluble nitrogen compounds, this was not reflected in leakage of intermediates: no radioactive substances, other than those fed, were ever detected in the media in which the tissues grew.

The effects of growth: increases of substance and activity. To make the comparisons between the fast-growing and the slow-growing tissue and to bring out the extent to which each tissue substance or quantity was accentuated by growth, the following procedure has been adopted. This will be illustrated first with reference to fresh weight.

At periods 1 and 5 the mean fresh weights of all the slow- and fast-growing tissues were obtained without regard to the different C^{14}-substrates added. This is justified because the individual treatments exerted little, if any, effect on the total fresh weight (see Table II). By taking the ratio of the mean fresh weight of the fast-growing tissues to that of the slow-growing tissues, the extent to which the increased growth produced by the stimulus of the coconut milk affected the fresh weight at periods 1 and 5 is shown. This general procedure has been repeated for the content of total protein, using the amount of protein alanine as the index of protein content. This is obviously justified in view of the constancy in the composition of the protein. Again the same procedure was repeated using total respiration as the quantity measured. The data from which the protein comparison was made are to be found in Tables

VI, VII, and VIII and the respiration data are to be found in Table II. The same form of analysis was applied to the total radioactivity present in each amino-acid of the protein which became appreciably radioactive. The data for these comparisons are obtained from Tables X and XII. All this information is summarized in Table XX.

TABLE XX

Relative increases in fresh wt., total protein, total respiration, and total radioactivity of the protein due to growth; i.e. ratio of the quantity in the fast- to that in the slow-growing cultures

	Period 1	Period 5	
Fresh wt.	2·9	3·7	
Total protein	2·9	3·7	
Respiration	4·3	5·1	
C^{14}-Activity in amino-acids of the protein			
Glutamic acid	—	5·6	
Aspartic acid	—	4·6	5·1
Threonine	—	5·2	
Proline	—	3·9	
Hydroxyproline	—	2·0	3·0

It is obvious from the comparison of the effects observed at periods 1 and 5 respectively that the overall effect of the growth stimulus increased with time, as was to be expected. The more interesting features are these. There was a remarkable similarity between the stimulus given by growth to the gain of fresh weight and of total protein; so much so that the ratios between fast- and slow-growing tissue for these quantities were identical at periods 1 and 5. This striking fact clearly means that the acquisition of water by the growing cells is not merely osmotic in nature but is closely linked to the metabolism with which protein synthesis is concerned. This is not an entirely new observation. Much recent work has laid stress on the possibility of non-osmotic and metabolically activated water intake by cells (Bennet-Clark *et al*, 1936; Frey-Wyssling, 1948), and indeed in earlier work on tissue slices some similar observations were made by one of us (Steward, Stout, and Preston, 1940). The striking parallelism between increased water intake of potato-tuber slices under conditions conducive to salt accumulation, their protein synthesis, their salt accumulation, and their aerobic respiration rate were all stressed, and it was clearly implied that the intake of water during these experiments was metabolically determined and characteristic of the cells ability to grow.

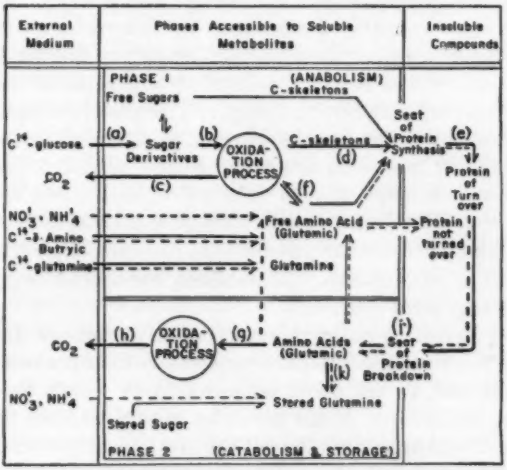
Thus, in these tissue cultures, stimulated to rapid growth, the increment of fresh weight should not be considered as a mere aspect of osmotic water absorption. Rather, the water is actually being built into the structure of the cell as an essential feature of its growth, similarly to the increment of total protein.

The next striking fact from the analysis of Table XX is that the stimulus to the growing cells accentuated total respiration *much more* than it accentuated the growth as measured by increments of fresh weight and of total protein.

The greater stimulus to respiration than to protein synthesis is shown by the ratios 4·3 : 2·9 and 5·1 : 3·7 for periods 1 and 5 respectively. This clearly means that in these tissue systems the respiration rate is not determined either by the total mass of protein or by the total mass of water in the cells. Alternatively, the efficiency of the protein in the fast-growing tissue in maintaining respiration is much greater than that of the slow-growing tissue.

The effect of the growth stimulus on the incorporation of carbon from the C^{14}-substrates into specific protein amino-acids, are of two kinds. In one group

Diagram to Illustrate the Main Metabolic Pathways in Cultured Carrot Tissue Explants



Pathways emphasized by the experimental treatments are indicated by heavy lines

FIG. 1

of amino-acids, represented by glutamic and aspartic acids and threonine, the stimuli to growth as shown by the ratios of the C^{14}-activity in the amino-acid proteins of the fast-growing tissue to those of the slow-growing tissue are similar to the stimuli to respiration in the faster-growing tissues. Treating these three amino-acids (glutamic acid, aspartic acid, and threonine) in one category, the accentuated incorporation of C^{14}-activity in the protein amino-acids of the fast-growing tissue over the slow-growing tissue at period 5 is 5·1. This is exactly the ratio which measures the increased stimulus to respiration in these tissues. Carrying out the similar analysis for hydroxyproline and proline, the value of the ratio which measures the accentuated incorporation, due to growth, of the C^{14} into these amino-acids in the protein is very much

smaller; this turns out to be close to that which measures the increased formation of *total* protein and the increased acquisition of fresh weight.

The explanation is as follows. Following Fig. 1 and the analysis set out in Part III, it is apparent that part of the respiration varies with the rate of protein 'turnover'. Thus part of the carbon dioxide which emerges from respiration originates from protein which is broken down, and the pace of alternating protein synthesis and breakdown will determine the extent of carbon dioxide emerging by this route, shown on Fig. 1 by the sequence (*i*), (*g*), (*h*). It is this pathway of respiration which is greatly stimulated in the fast-growing tissue.

The scheme also shows that the carbon involved in the synthesis of protein comes predominantly from sugar. Therefore, those amino-acids which incorporate their carbon primarily from sugar, by this pathway, can be used to measure the rate of turnover. Even though the carbon may have been derived from glutamine, it would merge with that from glucose *en route* to protein, following the pathways (*d*), (*e*) (*f*) of Fig. 1. Therefore, the significance of the similar behaviour of glutamic acid, aspartic acid, and threonine on the one hand and total respiration on the other is now obvious. The stimulus to growth increased the pace of that cycle by which carbon from sugar was incorporated in protein (eventually to emerge as carbon dioxide) to the same extent that it stimulated the incorporation of radioactive carbon from applied substrates in these amino-acids; this stimulus was definitely greater than the stimulus to total protein synthesis.

The last significant point of this analysis is as follows. Other evidence, (see Part III), has suggested that the proline was incorporated in the protein directly, in contrast to the other amino-acids in which the incorporation seems to be more indirect. Since the ratios which measure the accentuated incorporation of radioactivity in the proline and hydroxyproline of the protein in the fast- compared with the slow-growing tissues are very similar to the corresponding ratios which measure the increment of total protein and total fresh weight, it seems that these three quantities are all similarly determined. In other words, there is no reason to suppose that the proline and hydroxyproline of the protein participate specifically in that sequence of alternating synthesis and breakdown of protein which leads to the emergence of carbon dioxide.

This analysis, therefore, presupposes two types of protein, the one type being regarded as metabolically active protein and the other as metabolically inactive, possibly structural, storage, or enzymic protein. The protein in the tissue cultures which is rich in proline and hydroxyproline seems to belong to the latter category.

It is obvious that the proline rich, relatively inert, protein will also contain the other amino-acids, although probably in relatively lesser amounts, but these amino-acids will not incorporate activity at the same rate as their counterparts in the metabolically active protein. However, when analysing the cellular protein in bulk, these amino-acids from the inactive and the active

protein moieties are mixed and determined together. The presence of a protein moiety in which the amino-acids are apparently incapable of turnover will thus tend to lower the apparent turnover of the same amino-acids in the active fraction which is capable of turnover. Hence the increased incorporation of C^{14} into the protein amino-acids glutamic acid, aspartic acid, and threonine, shown in Table XX, might have been larger if it had been determined with reference solely to the 'active' protein moiety. Similar reasoning can be applied to the respiration which also has two moieties as shown on Fig. 1. One of these (the sequence *a*, *b*, *c*) may not be as much affected by the growth stimulus as the other (the sequence *a*, *b*, *d*, *e*, *i*, *g*, *h*).

GENERAL DISCUSSION OF THE RESULTS

Experimental design. This investigation is of a kind for which there are few precedents, particularly in the case of higher plants, because it recognizes and attempts to utilize all the complications due to growth and metabolism. The metabolism of the systems involved has been investigated while the tissues were maintained in a state of active growth, and it is the intervention in this metabolism of the C^{14}-labelled substrates, combined with the techniques of sterile tissue culture, of chromatography and radioautography, which made the investigation feasible. The value of the conclusions from this investigation emphasizes the need for the greater use of this sort of experiment.

However, even in the application of the C^{14}-tracer techniques it has been necessary to formalize somewhat the arithmetical treatment of the data which were obtained. The results clearly show that the mere detection of radioactivity may, of itself, be either of little value or even lead to error when one is attempting from such information to reveal the course of metabolism. In experiments involving C^{14}-labelled compounds there is need to measure the specific activities of the carbon compounds involved, but, to avoid still further difficulties, one should recognize that the intact cellular system is not homogeneous, but consists of a complex system of cytoplasm and its inclusions. The separate phases, or compartments, of this system may perform quite different reaction sequences even though they involve similar intermediates. Recognizing this complication, the data of specific activity must be so interpreted that they deal with the concept of the 'apparent specific activity of the carbon which is in transit through a given molecular species'. It is evident from the data that have been obtained that this is feasible, and the method of analysis adopted could be applied to a wider range of metabolic situations than has hitherto been done. Photosynthesis furnishes the dramatic example in plant physiology of the use of the C^{14}-techniques. Despite the fact that photosynthesis clearly involves a heterogeneous system in the cell, concepts and observations of the kind found necessary in this investigation have not been applied; if this were done still further information might be gained. In fact, reference to the long series of papers dealing with the path of carbon in photosynthesis will show that little, if any, use has been made of the

measurement of the specific activity of the postulated intermediates and the changes that occur with time (Calvin and Massini, 1952).

In the method of presentation here adopted there is little that is intrinsically new. What is novel is the numerical presentation of a wide range of data in such a way that unusual, or anomalous, events become immediately obvious so that they can be subjected to further analysis. Examining this body of data it was necessary, in order to determine metabolic sequences or pathways, to use the concept of the 'specific activity of the carbon moving through the metabolic pool' in addition to the specific activity of the substance in question. In this way, quite novel information was derived, such as the need to postulate the segregation of entering glutamine and glutamic acid, which may furnish nitrogen for protein synthesis, from the glutamic acid and glutamine that results from protein breakdown. Another conclusion that flows from this method of analysis is that the glutamic acid derived from entering glutamine cannot be immediately incorporated, as such, into protein. The sort of difficulty inherent in the use of specific activity data alone to interpret reaction sequences, becomes evident when it is seen that the specific activity of carbon in free glutamic acid in the cell may exceed the specific activity of the carbon in the glutamine from which it was clearly derived. All of these considerations reinforce the plea that data from radioactive tracer experiments need to be very carefully scrutinized before far-reaching conclusions on reaction pathways are drawn.

Experimental material. In the choice of experimental material for metabolic studies, many considerations apply. The widespread current emphasis on the use of micro-organisms, particularly bacteria, testifies to their obvious advantages. In fact, after the present investigation had been planned and largely carried out it was learned, with interest, that a comprehensive and somewhat similar investigation had been made on the metabolism of *Escherichia coli* by Roberts *et al.* (1955). Notwithstanding the value of the so-called comparative biochemical approach, differences between bacteria and higher plants do exist.

There is in higher plants a much more conspicuous internal aqueous phase, represented by the vacuole and perhaps even the cytoplasm, which contains far greater quantities of soluble metabolic constituents. Also there is in the bacteria a much greater tendency toward exogenous metabolism at the boundary surface between the cell and its medium. Whereas many bacteria, particularly the more strictly heterotrophic, will readily ferment glucose, they tend to pass the fermentation products largely into the external medium, so that labelling of the tissue constituents, such as protein, from glucose is thereby rendered difficult (Bidwell, Vittorio *et al.*, 1955). The tissue used in this investigation readily incorporates the carbon of sugar into the protein. In this respect the nitrogen autotrophic bacteria more nearly resemble the tissues of nitrogen autrophic higher plants. It may be that bacteria depend less upon the Krebs cycle as a source of energy than the higher plants (Roberts *et al.*, 1955).

Another complication exists in the use of bacteria for longer-term experiments involving growth. The growth-rates of bacteria are not long maintained and the cultures may pass rapidly through a sequence of changes, each with its own metabolic characteristics; whereas the tissue cultures here used may be maintained, for relatively long periods of time, in contrasted states of growth. Therefore the use of plant-tissue culture systems, as here described, has obvious advantages for the investigation of the contrasted metabolism of growing and non-growing cells.

Inasmuch as an original carrot-tissue explant, used in its relatively non-growing mature state, can be kept by tissue-culture techniques under aseptic conditions, and inasmuch as the tissue in its growing state produces a relatively large amount of homogeneous, randomly proliferating cells, the advantages of this sort of system over the tissue-slice technique for metabolic studies are apparent.

The dynamic state: proteins and respiration

The present investigation has profited by the now obvious idea that the essential physiological functions cannot be studied in isolation. This dilemma is inherent in all investigations concerned with the organized system, as distinct from the mechanism of individual reactions. The differences between the actively growing and the relatively inactive cellular states concern not so much the substances they contain, or the actual reactions they carry out, but rather the pace at which the whole dynamic machinery works. It seems quite essential, therefore, to study these processes in the organized system rather than isolating them for separate study, as in so much of current biochemistry. The present investigation has shown clearly that even such topics as respiration and nitrogen metabolism, water uptake, and protein metabolism should not be considered individually and apart from each other. In fact, one of the points of departure was an earlier recognition that in the cells of tissue slices the respiration and protein synthesis, salt uptake, and water absorption were in some way linked together (Steward and Preston, 1941).

While current thinking on intermediary metabolism definitely stresses a dynamic point of view there still is a tendency to consider the various aspects of metabolism separately. For example, current theories of glycolysis, the Krebs cycle, and the oxidative pathways to carbon dioxide do not necessarily presuppose the direct intervention of nitrogen compounds, or of nitrogen metabolism, to make the whole process work. It is true that, following Chibnall, Vickery, and others, the various points of departure from this carbohydrate metabolism which lead off into the channels of nitrogen metabolism have been stressed. This idea reaches its extreme development in the recent view that the Krebs cycle may be even more important as a synthetic mechanism than it is as the means by which energy is released (Krebs *et al.*, 1952; Roberts *et al.*, 1955). In other words, various 'ports of entry' for nitrogen in nitrogen metabolism have been generally recognized as intermediaries of the

carbohydrate metabolism. This, however, is very different from the recognition that the pace of respiration is maintained at a certain level by a similar pace of parallel reactions involving the nitrogen compounds. This latter point of view perhaps owes its genesis to Gregory and Sen (1937) who, when studying the metabolism of barley leaves subjected to various nutritional levels, concluded that the pace of what they called the 'protein cycle' determined, in large part, the pace of respiration. One of us contributed similar evidence and a similar point of view for the case of aerated potato-tuber slices (Steward, Stout, and Preston, 1940). The present investigation follows and enlarges upon the earlier ideas, recognizing a cycle of protein nitrogen synthesis and breakdown, which in its operation determines the rate of evolution of part of the carbon dioxide. In this 'protein cycle' sugar furnishes the carbon, but this only becomes available for respiration after prior incorporation into protein. If this point of view is substantiated, and it is believed that this investigation goes far to do this, it gives point to the elementary fact that the main reactions of cells only occur in a system predominantly composed of proteins capable of being metabolized.

The idea that proteins may serve as the respiratory substrate in starving leaves has long been recognized (Chibnall, 1939) and more recently reinvestigated (Krotkov, 1939). It has been shown that once the endogenous sugars of wheat leaves are exhausted by starvation, newly formed sugars in the leaves cannot serve as respiratory substrates (Bidwell, Krotkov, and Reed, 1952), indicating that the direct respiratory mechanism, proceeding through Phase 1 of Fig. 1, is permanently dislocated following starvation, even though the oxidative cycle via protein still operates as in Phase 2. It is of interest to note, in this connexion, that when protein breakdown occurs in darkened pea plants, there is a large accumulation of most amino-acids, but free aspartic and glutamic acids decrease in quantity (Miettinen, 1955). This appears to be a further consequence of the respiratory mechanism which utilizes the products of protein breakdown as substrate. Glutamic acid in particular has been shown to be important in this process.

Roberts *et al.* working with *Escherichia coli* (1955, p. 426) found that 'it is clear that some reaction sequence other than the Krebs cycle must be responsible for the large quantity of CO_2 produced' and they were unable to find a satisfactory source for the missing carbon dioxide. However, it is stated (p. 427) 'when nitrogen is omitted from the medium, the rate of carbon dioxide production drops to roughly one-half of the rate during growth'. These observations suggest that a comparable protein cycle exists in *E. coli*, in spite of their contrary claim (p. 433) that 'the dynamic state' as observed in adult tissues of higher organisms does not apply to these rapidly growing bacteria.

The idea of a protein cycle in respiration also leads to an explanation of certain otherwise inexplicable results. When C^{14}-glucose was supplied to wheat leaves, the production of unlabelled carbon dioxide increased, instead of decreasing, by competition between the exogenous labelled glucose and the endogenous substrate, as would be expected (Bidwell, 1954; Vittorio *et al.*,

1955; Krotkov and Risve, 1956). This, however, is readily explained if the fed glucose is supplying intermediates for protein synthesis, increasing the rate of protein turnover, and thus increasing the intensity of that portion of the respiration which utilizes protein breakdown products as substrate. Such products would, of course, be relatively unlabelled at first, due to the large mass of protein. The production of unlabelled carbon dioxide decreased with time (Bidwell, 1954), as the protein slowly became labelled from the fed glucose.

Growth and the protein complement of cells

Again, a point of departure of this investigation was the earlier recognition that the storage cells of potato tuber, which enter upon the active metabolism leading towards growth, salt accumulation, and protein synthesis, converted the soluble nitrogen compounds of the intact storage organ to a new complement which is more characteristic of the active cell; in this transfer, from one state to the other, glutamine was conspicuous. With the great increase of knowledge of transamination, transamidation, and group transfer reactions involving the γ-glutamyl linkage, the central role of glutamine now almost passes unchallenged. The importance attributed to glutamine and glutamic acid in the interpretation which has been made (see Fig. 1) is consistent with this.

It is also interesting that Vrba (1955), reviewing the metabolism of brain during muscular exercise, gives special prominence to 'a cyclical process of degradation and resynthesis of protein' and 'in this process free glutamine is formed from the free glutamic acid which is simultaneously an initial link for the phase of resynthesis'. The broad parallelism with Fig. 1 is clear.

It has long been, and still is, a problem to know what factors intervene to convert otherwise mature cells into the active proliferating state which is associated with cancerous or tumerous growth in both plant and animal systems.

Older views, recently revived (Warburg, 1956), held that proliferating, or cancerous, cells accentuated the glycolytic part of the respiratory system and thus obtained enough metabolically useful energy, in the form of ATP, to suffice for their growth. By contrast, normal cells only build up their store of usable energy from the oxidative respiratory cycle (cf. also Weinhouse, 1956, p. 17). It is natural to presuppose that systems so different in their ability to grow might even contain fundamentally different protein complements. The dramatic result of the present investigation is that the relative amino-acid composition of the protein is astonishingly similar even though the cells are capable of growth to a quite different extent. One has to seek, therefore, the differences in the evident ability of the rapidly growing system to synthesize protein, not so much in the *kind* of reactions that they carry out, but rather in some property that determines their pace. Amongst the many investigations that have been made on the nitrogen content of tumour and neoplastic tissue contrasted with normal tissue in the animal body, a recent investigation on kidney tissue is relevant. The differences between the two types of kidney

tissue are not concerned primarily with the amino-acid composition of the protein that they contain (Easty and Ambrose, 1955). Easty and Ambrose look for the differences rather in the sequence of the amino-acids in the molecule, arguing from differences in the stability or ease of hydrolysis of the protein from the two types of tissue.

It is interesting that the protein complement of carrot-tissue explants is now conceived to consist of two moieties, as illustrated in Fig. 1. One moiety, the 'inactive' protein, consists of protein which, when synthesized seems to be relatively quiescent, is not actively turned over, and may be a structural or storage protein although the possibility exists that it may have catalytic or enzyme properties. The remaining protein complement, designated 'active' protein in Fig. 1, is distinguished because it is in rapid turnover and is associated with respiration. As shown by the use of radioactive carbon, much of the carbon dioxide which emerges does so only after the carbon has passed from carbohydrate through this particular protein moiety. A similar view on the existence of two different protein types has been reached by Kylin (1953) working on wheat plants, and using an entirely different approach. Similarly it has been reported that the concentration of plant enzymes remains constant even when the content of other proteins changes drastically, and may even remain constant during ontogeny (Axelrod and Jagendorf, 1951; Wood and Sibley, 1952). More recently, it has been shown that at least two different types of protein with widely different rates of synthesis and decay (i.e. turnover) are present in the tobacco leaf (Dormer *et al.* 1957), as well as in animal tissue (Simkin and Work, 1957).

Looking at the problem of the proliferating as contrasted with the resting cell, it now seems that the main differences between these two states is that the proliferating cell has its rate of metabolic turnover, i.e. synthesis and breakdown, of the more mobile protein complement very much accentuated; whereas the proteins of the resting cell remain much more in the quiescent condition. By contrast with the coconut-milk growth promoting factors, which stimulate cells to grow and promote the synthesis of a kind of protein that may readily be reused in metabolic cycles, the supposed effect of a virus is to promote the formation of a type of protein which constitutes a 'point of no return'. *These two different stimuli therefore seem to operate on the two different aspects of protein metabolism and synthesis.*

The only evidence yet obtainable concerning the difference between the more active protein and the more quiescent protein fractions is that the protein of the resting potato tuber and the protein of the resting carrot root, either contains no hydroxyproline, or only a very small amount (Steward and Thompson, 1954). By contrast the protein of the growing cultures contains hydroxyproline in greater amount. It remains, however, for future work to say what the significance of this observation might be. It is interesting that hydroxyproline is especially associated with collagen, and that stimulus to collagen synthesis is associated with various situations in the animal body and bacteria in which cell division is accentuated.

Phases in cells. An integrated view of the carbohydrate and nitrogen metabolism of the cell as a whole emerges from this work; this view takes account of the segregation of cells into distinct phases. The familiar processes of carbohydrate breakdown and oxidation through the Krebs cycle leading off, through the acceptance of nitrogen by appropriate carbon residues, to protein synthesis seems to occur in some distinct location in the cell which has been designated as Phase 1 (see Fig. 1). It is also clear that protein breakdown, leading off through processes of oxidative degradation and deamination to carbon dioxide and yielding predominantly glutamine as a temporary storage reserve of nitrogen, seems to be in a separate phase of the cell (Phase 2, cf. Fig. 1). The extent to which Phases 1 and 2 might be identified with mitochondria, or the aqueous part of cytoplasm, is of course not known, but their spatial separation is required by the interpretation of the C^{14}-labelling experiments.

From time to time certain anomalies have been reported which may be explained by the idea of compartments or phases in cells. Roberts *et al.* (1955) have reported that certain amino-acids (notably threonine and lysine) when supplied exogenously to *Escherichia coli* enter completely different sequences of reactions than do the threonine and lysine already within the cells. Similarly, Vittorio *et al.* (1954) have shown that C^{14}-labelled glucose supplied to tobacco leaves apparently undergoes different reactions from the endogenous glucose. Much has been said about the unavailability of recent products of photosynthesis for respiration and it has been suggested that the Krebs cycle is inoperative in light (Calvin and Massini, 1952), in spite of the fact that its ability to operate is apparently not affected by light (Bidwell, Krotkov, and Reed, 1955). All these examples may be understood on the basis of phases within the cells: exogenously supplied compounds or recent products of photosynthesis may enter a phase or reaction sequence separate from that encountered by their endogenous counterparts.

Still more recently Cowie and Walton (1956) have investigated protein synthesis in *Torulopsis utilis*. Cowie refers to the 'pool' as that material which is immediately available for protein synthesis and concludes that 'pool amino acids are not free amino acids but are adsorbed to larger molecules and these adsorbtion sites are intimately connected with the process of protein synthesis'. The same amino-acids may also exist elsewhere in the cell in a more generally accessible state. This interesting view attempts to interpret *T. utilis* in a manner not unlike the present interpretation of carrot cells. Whereas Cowie's 'pool amino acids available for protein synthesis' could be homologized with our carbon skeletons at the point of condensation, their origin is differently conceived. Cowie's data are compatible with entry of exogenous amino-acids, binding on large molecules, and thence direct incorporation into the protein. His data do not require that the carbon of amino-acids entering the cell must pass through the Krebs cycle *en route* to protein.

The possible existence of compartments in cells has also been suggested recently by Jardetzky and Barnum (1958) on the basis of the mathematical

analysis of tracer experiments, and they have been demonstrated pictorially in chloroplasts of *Euglena* by Sager (1957). The free sugars of chloroplasts have also been shown to differ qualitatively and quantitatively from those in the rest of the cell (Heber, 1957), indicating that the chloroplast itself may represent a compartment of the type envisaged. Another example is furnished by Rautanen and Tager (1955) who have demonstrated the existence in oat seedlings of two distinct L-glutamic acid dehydrogenases, one soluble and DPN dependent and the other associated with cytoplasmic inclusions and relatively independent of DPN; these observations are compatible with the existence of glutamic acid in different locations in the cell, such as the Phases 1 and 2 of Fig. 1, in which it participates in different kinds of metabolism.

Substrates of protein synthesis: the present investigation

The fundamental question—where does protein synthesis occur in the cell?—remains outstanding. Much current thought is directed toward the fact that protein synthesis may occur in protoplasmic inclusions or granules, such as mitochondria. Since the present investigation relates to the intact cellular system, it does not bear directly on this question. However, the important point which emerges is that one must visualize the protein synthesis occurring at some seat, or surface, to which carbon residues and mobile forms of nitrogen are supplied and it seems necessary to recognize that the combination of carbon residues, their acquisition of nitrogen, and the furnishing of the energy necessary for protein synthesis all occur compactly at this seat of protein synthesis. The point here is that if amino-acids, e.g. glutamic acid, are to be regarded as intermediates in this sequence, they do not mingle with the amino-acids which are generally dissolved in the aqueous phases of the cell.

Earlier, when interpreting the effects of cations on respiration and protein synthesis in potato slices (Steward and Preston, 1941) it was necessary to record that the salt effects were 'exerted in centres accessible to salts being absorbed' rather than to the ions already within the cell. This is also compatible with the concept of a discrete centre or surface at which protein synthesis occurs, this centre being remote from the phase of the cell in which soluble compounds (amino-acids and inorganic ions) are stored. In a similar way, Roberts *et al.* (1955) have observed that inorganic sulphate entering the bacterial cell is more readily incorporated into the protein than into the free sulphur-containing amino-acids.

In other words, carbon residues and sources of nitrogen, whether derived by reduction of nitrates, or supply of ammonia from the external solution, or by the oxidative deamination from the products of previous protein breakdown, must all be supplied to the seat of protein synthesis together with the energy necessary to bring about the condensation at the synthesizing surface. The requirements for this condensation and the nature of the synthesizing surface do not follow from the experiments, but there is no need to dissent

from the prevalent view that it acts like a template surface composed largely of pentose nucleic acid.

It should be stated, however, that the particular concept here developed of the synthesis of protein, followed by its breakdown, should be held to apply only to that part of the protein which is in an active state of synthesis and degradation. The remainder of the protein, which does not turn over, may be synthesized by an alternative route or different mechanism. The very fact that some amino-acids, e.g. proline and the derived hydroxyproline, in which these proteins seem to be rich, appears to be embodied intact from the soluble phases of the cell indicates that this entire mechanism of protein formation may be different.

Substrates of protein synthesis: interpretations of other experiments

There are several points of evidence in favour of the mode of protein synthesis here developed in contrast to the polypeptide hypothesis, i.e. the direct incorporation of free amino-acids. It is again evident in the present experiments that the concentration of soluble amino-acids in the cell in no way reflects the amino-acid composition of the protein, nor are the specific activities of those amino-acids, which are characteristic of the 'active' protein, related as they exist free and in the protein. For instance, the specific activities of protein-bound aspartic and glutamic acids are almost identical while their soluble counterparts are quite different (cf. Tables IX and X, XI and XII). A second point arises from other experiments in which C^{14}-labelled glucose and glutamine were supplied to isolated wheat leaves in the dark (Bidwell, Krotkov, and Reed, 1955). In spite of the fact that free glutamic acid was much more heavily labelled from C^{14}-glutamine than from C^{14}-glucose, more activity entered the protein glutamic acid from the supplied glucose than from the glutamine. Also, as in the present experiment, the proteins were more heavily labelled throughout from glucose than from glutamine. All these results indicate, at least for the compounds named, that free amino-acids do not serve as immediate building blocks of protein, but they are themselves formed in the process of protein synthesis.

When ammonia is supplied to a fungus (MacMillan, 1956) extensive amino-acid synthesis takes place. However, when glucose is also added, not only are amino-acids formed but protein is synthesized. This is compatible with the idea that glucose, not amino-acids, provides the carbon for protein synthesis.

The results of similar experiments to our own which were carried out on *Escherichia coli* by Roberts *et al.* (1955) are of interest. When one group of labelled amino-acids was supplied (arginine, leucines, and proline), they were incorporated directly into the protein without dilution and with little, or no, distribution of their activity. However, when glutamic acid, aspartic acid, alanine or threonine were supplied as labelled substrates, activity was found *at about the same level* in several protein amino-acids, including the one

supplied. This suggests equilibration in the soluble pool before incorporation of the carbon of the last-named amino-acids, and renders unlikely their direct incorporation into protein. Also, when labelled aspartic or glutamic acids were supplied, certain compounds (*notably proline and the basic amino-acids*) achieved *higher specific activity in the protein than did the supplied compound.* This indicates that proline and the basic amino-acids in the protein were derived from the supplied aspartic or glutamic acid by a more direct route than was the protein-bound aspartic or glutamic acid itself. All these results point to the fact that there are two different pathways for the incorporation of amino-acid carbon into the proteins of *E. coli* as has been shown also for the carrot tissues. The one route, for aspartic and glutamic acids and probably others, proceeds via deamination and subsequent progress through the Krebs cycle, and the other, involving proline and the basic amino-acids, is by direct incorporation into the protein.

These workers point to a paradox in their results, namely that free amino-acids may diffuse into, or out of, the cell with great ease, but endogenously formed amino-acids apparently cannot leave the cell prior to their incorporation into protein. They have resolved this paradox by visualizing the free amino-acids attached to some non-diffusable binding group, 'R'. It will be seen at once, however, that the paradox disappears if the free amino-acids are considered, not as protein precursors, but as breakdown products, the actual protein precursor being the carbon skeleton, together with ammonia or a suitable nitrogen donor. It is well known that this type of intermediate is present in cells in very small quantities undergoing very rapid turnover (i.e. it is in great demand at reaction sites), and hence would not be expected to diffuse out of the cells.

Some recent experiments with C^{14} on wheat plants by McConnell (1957) are of interest. Wheat grains were made radioactive and the specific activity of various cellulose components and respired carbon dioxide was determined. The respired carbon dioxide had a higher specific activity than any cellular component except the glutamic and aspartic acids in the protein, from which it must, therefore, have been derived. McConnell also concluded that the carbon of glutamic acid enters the protein after passage through the Krebs cycle. In experiments with *Pseudomonas*, Kornberg (1956) showed that 'most of the carbon of the amino-acids synthesized (in the protein) passes through the stage of carbon dioxide or compounds readily in equilibrium with carbon dioxide'.

Thus the consideration of results obtained with diverse materials by different investigators now lends support to the main thesis here developed. This thesis is that the metabolic pathways of respiration and protein metabolism are closely interlocked in actively metabolizing, growing, cells, and this occurs in such a way that the carbon of the respired carbon dioxide has been more recently incorporated in protein than conventional views on glycolysis and the oxidative carboxylic acid cycles would alone indicate. From these diverse observations the following main conclusions follow:

1. Protein synthesis and breakdown occur at specific sites in the cell. The carbon of sugar is more accessible to the site of synthesis than the carbon of many of the free, stored, amino-acids (e.g. glutamic acid, aspartic acid, threonine) which, as they exist compartmentalized in the cell, are to be regarded as products of breakdown and as storage products and not as the *immediate* precursors of protein. As the nitrogen of these compounds is redrawn into the cycle of protein synthesis, their carbon is respired away and is replaced in the protein by carbon skeleton drawn from sugar.

2. Stimuli to rapid growth and cell division accentuate total protein synthesis and, in the same ratio, the accretion of water by the cell which is therefore regarded as an active process and an integral part of growth. The carbon of some amino-acids (proline and hydroxyproline) enters protein directly, and the ratio of their incorporation in the protein becomes a measure of the increase of total protein, because these amino-acids are combined in a form such that they do not participate in metabolic turn-over. In addition, however, the stimuli to growth also promote the rates of 'turn-over' of metabolically active protein, and this results in increased incorporation of C^{14} in those protein amino-acids which participate in this cycle of synthesis and breakdown, and which draw their carbon into the protein from sugar.

3. Thus the rate of passage of C^{14} from sugar to carbon dioxide becomes a function of the pace of a reversible cycle of protein synthesis and breakdown. Therefore, to interpret the metabolism of growing cells, it is necessary to recognize that there are both metabolically active and inactive protein moieties, and that the carbon of the soluble metabolites participates to a greater or lesser extent in respiration or in protein synthesis according to their location in compartments in the cells. Light has been thrown on the contrasted behaviour of solutes in different compartments and of substances which are of endogenous or exogenous origin by the consideration of the specific activity of their carbon and the changes which occur with time.

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Non-hormonal Growth Promotion Shown by Aqueous Extracts

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SUMMARY

Bioassay of chromatograms of aqueous potato-sprout and -tuber extracts and lettuce-seed extracts, with the coleoptile, straight-growth test, indicates the presence of a growth promoter running near to the position of IAA and giving a spray colour reaction with Ehrlich reagent similar to that of IAA. It is shown that both promotion and spray colour are due to sugars. It is concluded that major nutrients may give large growth effects in this bioassay and that in certain cases ether extracts may contain quantities of these large enough to give rise to growth effects.

INTRODUCTION

SINCE the application of paper chromatography to the separation and estimation of growth substances in plant extracts a large number of investigations have been carried out, mainly on substances present in ether extracts. However, non-ether extractable growth substances have been shown to occur in aqueous residues following ether extraction (Housley and Bentley, 1956; Britton, Housley, and Bentley, 1956; Housley, Booth, and Phillips, 1956.) Such aqueous extracts will also contain some quantities of sugars, amino-acids, and other substances of which some are known to be active in the coleoptile test. During the course of an investigation into developmental changes in growth substances in the potato plant and in lettuce, using the techniques of coleoptile straight-growth assay and tests with spray reagents, evidence was obtained that in some extracts major nutrients may play a large part in the production of growth effects.

MATERIALS AND METHODS

Plant materials were ground with solid carbon dioxide and extracted with various solvents at 0–3° C. in the dark. At least 10 ml. of solvent were used per gram fresh weight of material and extraction in any one solvent was continued for a period of 24 hours, during which time three changes of solvent were made. Potato extracts were made with ether and water as solvents and lettuce-seed extracts with ether followed by 50 per cent. ethanol. Etiolated sprouts of Majestic, Epicure, and King Edward and tubers of Golden Wonder potatoes and seeds of *Gotte-à-forcer* lettuce were used. In some of the earlier work the aqueous extract was evaporated to a small volume under reduced pressure at 30° C. In later work it was freeze-dried to a small volume. The extracts were stored at −15° C.

Chromatograms were line-loaded for bioassay and, in general, spot-loaded for the determination of spray reaction. Descending chromatography in all-glass tanks was carried out with the following solvents: *iso*propanol/0·15 N. ammonia (80:20), distilled water, butanol saturated with water, and *n*-butanol /acetic acid/water (2:1:1). The spray reagents used were Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) for indole derivatives, aniline phthalate and *p*-anisidine hydrochloride for sugars, and ninhydrin for amino-acids. Sprayed chromatograms were developed in an oven at temperatures up to 80° C. or in a stream of hot air from an industrial air dryer.

Bioassay was by the straight-growth test using oat or wheat coleoptiles. The developed chromatogram was dried, cut into ten equal transverse segments and each segment eluted in 5 ml. of water. Ten coleoptile sections 10 mm. in length were placed in each eluate and the dishes were allowed to stand in the dark at 26·5° C. and the length measured after 16–20 hours. Butanol, when used, interfered with the test and therefore had to be removed; the following method was used. Each transverse segment, corresponding to one-tenth of the chromatogram, was rolled to form a cylinder which was then stood on end in the petri-dish in which it would ultimately be eluted. One ml. of water was run in, sufficient to thoroughly moisten the paper, and the paper was then dried in a stream of warm air not exceeding 30° C. in temperature. This process was repeated and the butanol was removed.

RESULTS

Chromatography of aqueous and ethanolic fractions was initially carried out in *iso*propanol/ammonia and a representative selection of the results of bioassay is given in Fig. 1. Growth promotion is shown in each case and tends to lie between Rf 0·3–0·4. The results of spray tests are given below the histograms. Growth promotion is correlated with a purple spray colour with *p*-MeAB and a yellow colour with *p*-anisidine. The purple colour with *p*-MeAB is similar to that given by IAA; that it is not due to IAA is shown by adding IAA to the extract; on chromatography there was a separation between the colour due to the extract and that due to synthetic IAA. Chromatography in distilled water did not separate the yellow *p*-anisidine colour from the purple *p*-MeAB colour and the region of growth promotion on bioassay remained associated with the colour zone.

Comparison of Rf's in *iso*propanol/ammonia showed that sugars (sucrose 0·30, glucose 0·36, fructose 0·39) would lie within the zone of growth promotion and the yellow colour with *p*-anisidine indicates that they are present in the extracts. The purple *p*-MeAB colour might be due to tryptophane which has an Rf of 0·28 in this solvent. However, a fact not usually mentioned in accounts dealing with the spray reactions of plant extracts is that common sugars give a colour reaction with *p*-MeAB. The colour given is purple similar to IAA or tryptophane but more heat is required to develop it. Activity curves of sucrose (glucose and fructose behave similarly) and tryptophane are given

in Fig. 2. Maximum promotion by both compounds is equivalent to that given by 0·1 mg./l. IAA. Growth promotion by tryptophane and sucrose was shown to be due to those respective compounds and not to contaminants, by chromatography followed by spray tests and bioassay. It was noted that both compounds had appreciably higher Rfs in *iso*propanol/ammonia when they were chromatographed from a line as against a spot loading. A chromatogram

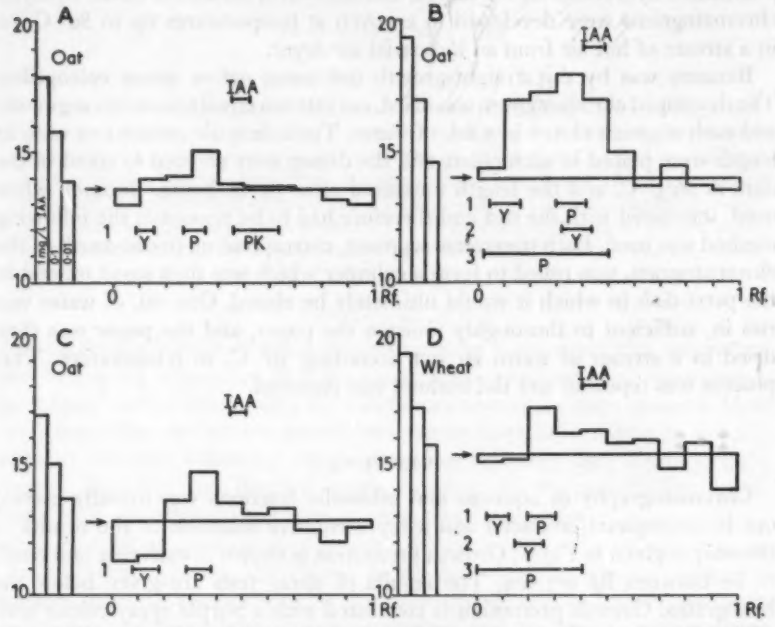

FIG. 1. Chromatographic analysis of aqueous extracts run in *iso*propanol/ammonia. A. King Edward 5 g. fresh wt., B. Majestic 50 g., C. Golden Wonder 6·5 g., D. *Gotte-à-forcer 2 g.* Spray reagents: 1. Ehrlich; 2. *p*-anisidine; 3. ninhydrin; Colours. Y = Yellow, P = Purple; PK = Pink; ordinates show extension growth of coleoptiles in mm. Abscissae Rfs of chromatogram strips.

of a mixture of sucrose and tryptophane in optimum supply run in *iso*propanol/ammonia gave the interesting result (Fig. 3) that promotion by the two substances together is greater than that of either alone and is greater than that given by 1 mg./l. IAA. This histogram is closely similar to that of the aqueous extract of 'Majestic' sprouts when it is overloaded on the chromatogram (Fig. 1*b*).

Chromatography in butanolic solvents gave a better separation between sugars and tryptophane. Spray reactions shown by 'Golden Wonder' tuber extracts run in butanol/water indicated that the purple *p*-MeAB colour was due to sugars alone. Two purple zones were recorded in lettuce extracts run in butanol/acetic acid/water and comparison of spray reaction with bioassay

showed that promotion occurred in the sugar region. The question which now arises is whether there is sufficient sugar in the extracts to account for the observed promotion. In the case of potato 50 g. fresh weight of sprouts would contain between 50 and 500 mg. of sugar (Appleman, 1916) and bioassays of 50 mg. of sucrose eluted in 5 ml. give considerable promotion, suggesting that the promotion of the aqueous extracts can be explained in terms of

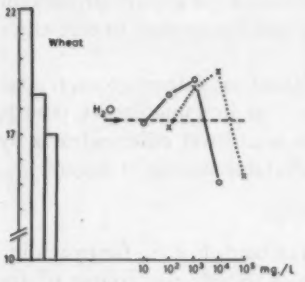

FIG. 2.

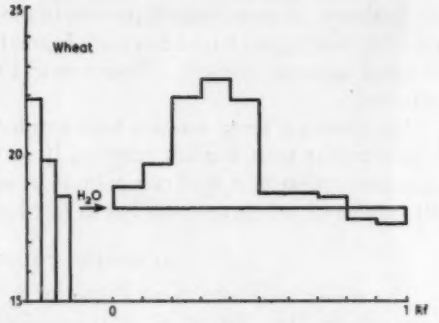

FIG. 3.

FIG. 2. Activity curves of sucrose (. . . .) and tryptophane (——). Concentrations shown as abscissae. Wheat coleoptile extension as ordinates (mm.).

FIG. 3. Chromatography of sucrose (50 mg.)+ tryptophane (5 mg.) run in *iso*propanol/ ammonia. Wheat coleoptile extension shown as ordinates (mm.).

sugars. Sugar determinations made on hydrolysed chromatogram eluates of 'Epicure' sprout extract gave a value of 200 mg./50 g. fresh weight.

DISCUSSION

In the above experiments sucrose gave consistent promotion with assays of less than 24 hours. Other workers (Bentley and Housley, 1954) have found that sucrose had little effect in assays of this length but that promotion was given in assays of 48 hours or more. One explanation of this difference in results which can be given is that coleoptiles vary in their response to sucrose. Variation could be a function of the variety used or may be due to storage or seasonal differences (Tyce, 1957). On the other hand, in the present experiments both tryptophane and sucrose have been assayed with Victory oats and Pilot wheat at most times of the year and promotion is given in each case. Atle wheat, used in a few tests, gave a far greater response to sucrose and in fact with 1 per cent. sucrose showed greater elongation than with 1 mg./l. IAA.

Non-hormonal substances may be responsible for, or contribute to, the growth promotion shown in certain ether extracts. Enough sucrose was shown to be extracted by ether from sucrose solution to give considerable promotion if the ether was not dried prior to chromatography. In a solvent such as *iso*-propanol/ammonia in which sugars run close to the position of IAA, fairly

small amounts of sugar, though not directly giving promotion, may exert an effect through synergism with the natural growth substance.

Comparison of the above results dealing with growth promotion by sugars and other nutrients can only be made with results of earlier experiments in which chromatogram elution has been carried out with water alone. Although promotion in the aqueous extracts reported here appears to be due almost entirely to sugars it is not suggested that this is generally or always true. Nevertheless as some sugar is present in most aqueous extracts it would seem possible that sugars have been responsible for some of the growth promotion in other aqueous extracts. These remarks may also be applied to wet ether extracts.

The results of spray reaction have a wider application. Sugars give a typical indole colour with Ehrlich reagent. It may be that in some earlier reports characterization of indole compounds in aqueous and wet ether extracts by Rf value and colour reaction has in fact been characterization of sugars.

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